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Steroid Receptors in Human Prostate

by



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A THESIS

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ABSTRACT

Human prostate hormone receptor assays are being evaluated in many laboratories, with a view to the use of such assays in the clinical management of prostate cancer, as is now the case in the management of breast cancer. Using an hydroxylapatite assay and the known receptor stabilizer, sodium molybdate, androgen (AR), estrogen (ER) and progesterone (PgR) receptor levels were measured in the cytoplasm of all three types of prostate tissue (normal, hyperplastic and cancerous) and nuclear AR was measured in normal and hyperplastic tissue (BPH).

A method for the isolation of purified nuclei (relatively free of cytoplasmic contamination) was established. A chromatin-depleted, salt-insoluble, proteinaceous intranuclear structure known as the nuclear matrix was isolated from these nuclei. Nuclear-extractable (or KCl-soluble) AR was measured and the nuclear matrices were assayed for the presence of AR (KCl-insoluble, or matrix-bound AR), in BPH and normal tissue.

Cytoplasmic AR was present in all three prostate tissue types examined, in comparable concentrations (mean values of 606 fmols/g for normal; 595 fmols/g for BPH; 646 fmols/g for cancer). ER was present in all three normal tissues (mean concentration of 335 fmols/g), in eight of 15 BPH specimens (mean concentration of 290 fmols/g), and in four of six cancer specimens (mean concentration of 440

fmols/g). PgR was found in all three normal tissues (mean concentration of 745 fmols/g), 13 of 15 BPH tissues (mean concentration of 1446 fmols/g) and four of six cancer specimens (mean concentration of 1806 fmols/g).

Nuclear extractable AR was found in all BPH specimens examined (mean concentration of 252 fmols/g) and in two of the three normal tissues. Nuclear matrix-bound AR was found in all BPH specimens (mean concentration of 574 fmols/g) and in all three normal tissues (mean concentration of 838 fmols/g).

The unequivocal demonstration of ER in BPH tissue, not previously reported, supports the proposal of a role for estrogen in the pathogenesis of this condition. The presence of ER in a proportion of malignant tissues also raises the question of the validity of routine exogenous estrogen administration to patients with advanced prostate cancer.

Since the cell nucleus is the site of action of steroid hormones, it is felt that assays for such nuclear steroid hormone receptors may indicate the steroid dependence of a tissue more accurately than cytoplasmic receptor assays. The relative value of salt-extractable and matrix-bound AR as an index of hormone dependence remains to be determined.

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CHAPTER 1

INTRODUCTION

The prostate is a chestnut-sized gland (Gray, 1973), situated distal to the bladder neck where it surrounds the commencement of the urethra (Figure 1). The gland is composed of numerous acini surrounded by a dense but variable fibro-muscular stroma. The acini are lined by cuboidal epithelial cells, and empty into the floor of the proximal prostatic urethra via 12-20 small excretory ducts. The gland is divided into lobes or regions designated as anterior, central and peripheral regions, with a smaller periurethral zone surrounding the proximal part of the prostatic urethra (Figure 2).

The prostate among mammalian organs shows extreme variation in structure (McNeal, 1981) and even within the order of primates, significant interspecies differences have been noted on gross and microscopic examination. As a result of this diversity, the existence of an adequate animal model for the study of human prostate disease has not yet been firmly established (McNeal, 1981). This fact must be borne in mind when extrapolating results from animal studies to the human.

The prostate is one of the largest glands in the body with unknown physiological function (Coffey and Isaacs, 1981). It is known that it supplies components of the

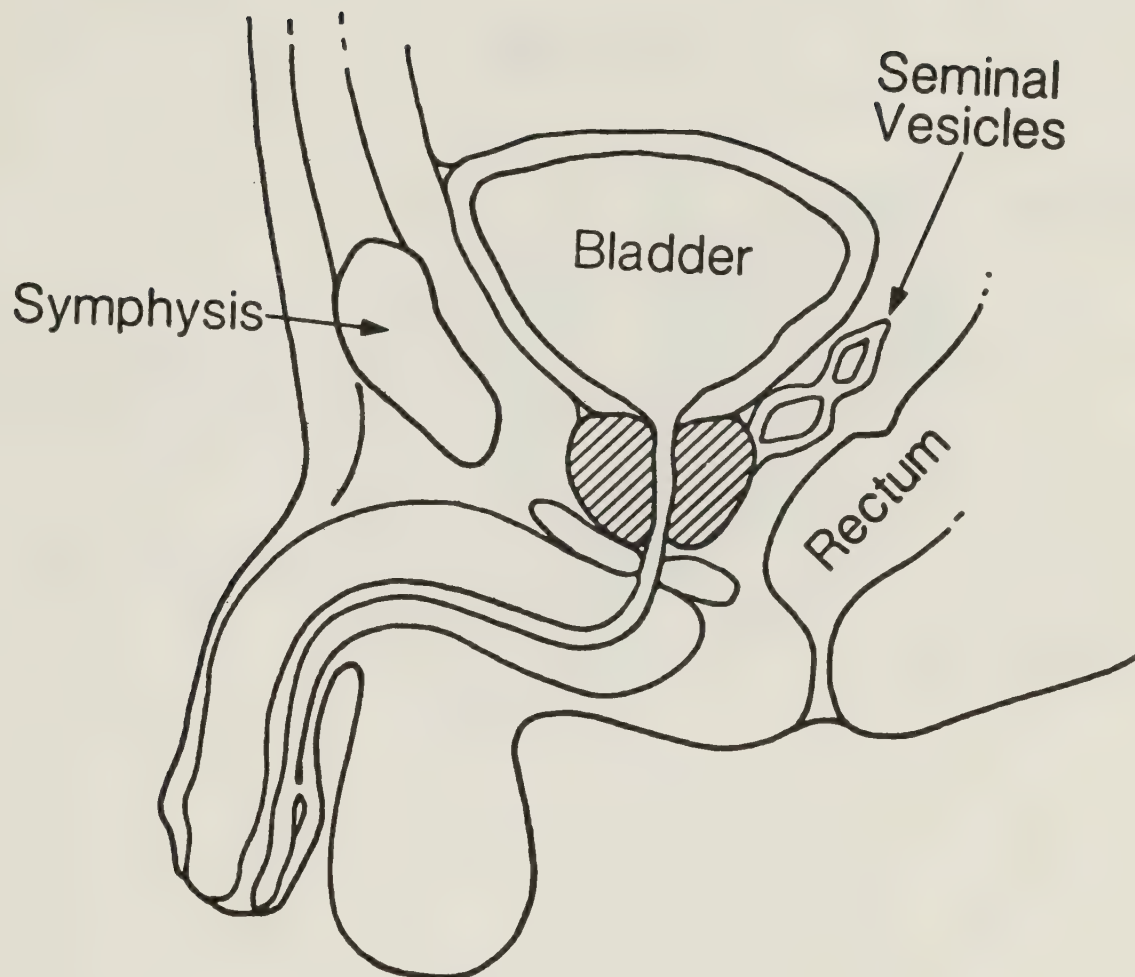


Figure 1. Anatomic site of the human prostate. Diagrammatic representation of a cross-section of the human male pelvis, showing the location of the prostate (shaded).

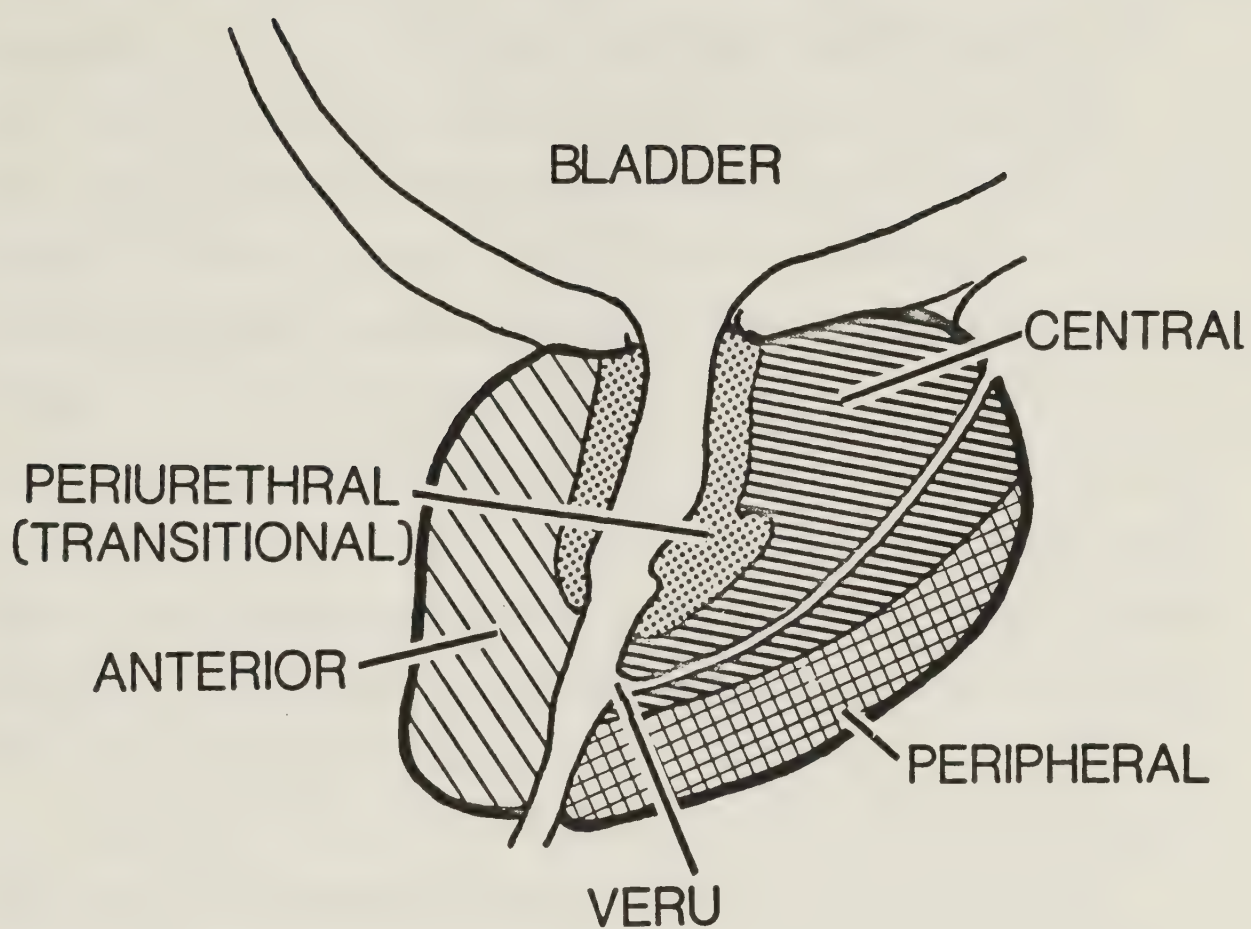


Figure 2. Anatomical regions of the human prostate.

ejaculate, but their biological function is not known (Coffey and Isaacs, 1981).

From the point of view of clinical significance and management, the prostate may manifest two disease entities, benign prostatic hyperplasia (BPH) in the periurethral zone and carcinoma in the peripheral region. The management of BPH, when symptomatic, is achieved through surgery and this is the only management in use currently. The surgery includes transurethral resection (TUR) with a loop electrode and accounts for the vast majority of prostatectomies (Grayhack and Sadlowski, 1976). The remainder of BPH cases are dealt with by open surgery, the adenoma being enucleated. With both of these methods, the prostate capsule and peripheral lobe tissue (to a greater or lesser extent) is left behind. Thus it is possible to observe the occurrence of carcinoma in patients who have had previous prostatectomy for benign disease, as the peripheral zone is usually the site of development of cancer.

Carcinoma is managed by surgery, radiotherapy, chemotherapy or hormone therapy, or a combination of these treatment modalities (Catalona and Scott, 1978). Surgery is indicated in two main instances: if the malignancy is confined to the gland, many surgeons carry out a radical total prostatectomy (removing the entire gland with its capsule) in the hope of completely eradicating the disease; on the other hand, if a malignant gland is

causing obstructive symptoms and a radical prostatectomy cannot be carried out, then a TUR is indicated to relieve the obstruction. Other surgical approaches include orchidectomy, adrenalectomy and hypophysectomy and these will be discussed under the heading of hormone therapy.

Some clinicians are once again using radiotherapy, (either external beam or local implantation of radioactive seeds) in the management of prostatic cancer (Ray et al, 1973; Bagshaw, 1975; Sewell et al, 1975; Whitmore et al, 1974). The local results to date are encouraging, and this mode of therapy has a definite role to play in prostatic cancer therapy (Catalona and Scott, 1978; Klein, 1979). Chemotherapy has not yet been shown to contribute significantly to the management of prostatic cancer, although much work is being done in this regard at present (Lamm and Sarosdy, 1982).

A major advance in the treatment of prostatic cancer occurred in the 1940's as a result of the Nobel Prize-winning work of Huggins and Hodges (1941), who demonstrated the clinical effectiveness of estrogen therapy for the disease. This endocrine therapy may be carried out by additive or ablative approaches: additive therapy can be performed by the administration of exogenous estrogen; for ablative therapy orchidectomy removes the source of androgens. Adrenalectomy and hypophysectomy are occasionally carried out to remove any extra-testicular

androgens or the source of prolactin and other pituitary hormones (Scott and Schirmer, 1962; Banalaph et al, 1974; Catalona and Scott, 1978). Between 60-80 per cent of patients with prostate cancer respond to some extent to either exogenous estrogen treatment, or orchidectomy (Resnick and Grayhack, 1978; Fergusson, 1972; Walsh et al, 1976). Exogenous estrogen therapy is by far the commonest form of treatment currently in use for prostatic cancer (Griffiths et al, 1979).

In the normal setting the differentiation of the prostate during embryogenesis, the growth of the gland at the time of sexual maturation and the formation of the prostatic secretions in the mature adult are all under the control of testicular androgens (Huggins, 1946-47; Coffey, 1978). Interestingly, neither cancer nor benign hyperplasia develops in castrated humans (Moore, 1944). It is assumed that the response to endocrine therapy occurs because the prostate is a steroid target organ, dependent on androgens for normal development and maintenance; thus tumours arising in the prostate may exhibit a steroid dependence similar to the parent tissue and tumor growth may be controlled through manipulations of the hormonal milieu.

It is generally accepted that the anti-androgenic effect of estrogen on the prostate is indirect, and occurs via the hypothalamo-pituitary axis (Coffey and Isaacs, 1981; Griffiths et al, 1979). The estrogens are thought

to 'feed-back' through this axis to decrease luteinizing hormone (LH) release and thereby reduce testosterone secretion (Alder et al, 1968). However, recent work using tissue cultures of human prostatic cancer cells has shown that estrogen may also have a direct effect on the prostate in some cases (Hudson, 1981). These findings support earlier reports of a direct effect of estrogens (Farnsworth, 1969; Harper et al, 1970).

Testosterone is secreted by the testes where it is manufactured by the Leydig cells from acetate cholesterol (Figure 3). The rate of testosterone production is under the control of LH released from the anterior pituitary and subject to feed-back control by free testosterone (Coffey and Isaacs, 1981). Testosterone serves as a pro-hormone for the formation of two types of active metabolites - dihydrotestosterone (DHT) and 17- β -estradiol (Wilson, 1980) (Figure 4). Ninety-five per cent of testosterone in normal human males is of testicular origin, the normal serum testosterone level being 600 ngm per 100 ml (Wilson, 1980; Coffey and Isaacs, 1981). Testosterone is transported throughout the body bound to plasma proteins as only approximately two per cent exists in the free form. The free portion of testosterone interacts with its target organs.

DHT formation occurs in part peripherally, but the major conversion takes place intracellularly in the prostate and seminal vesicles (Wilson, 1980; Coffey and

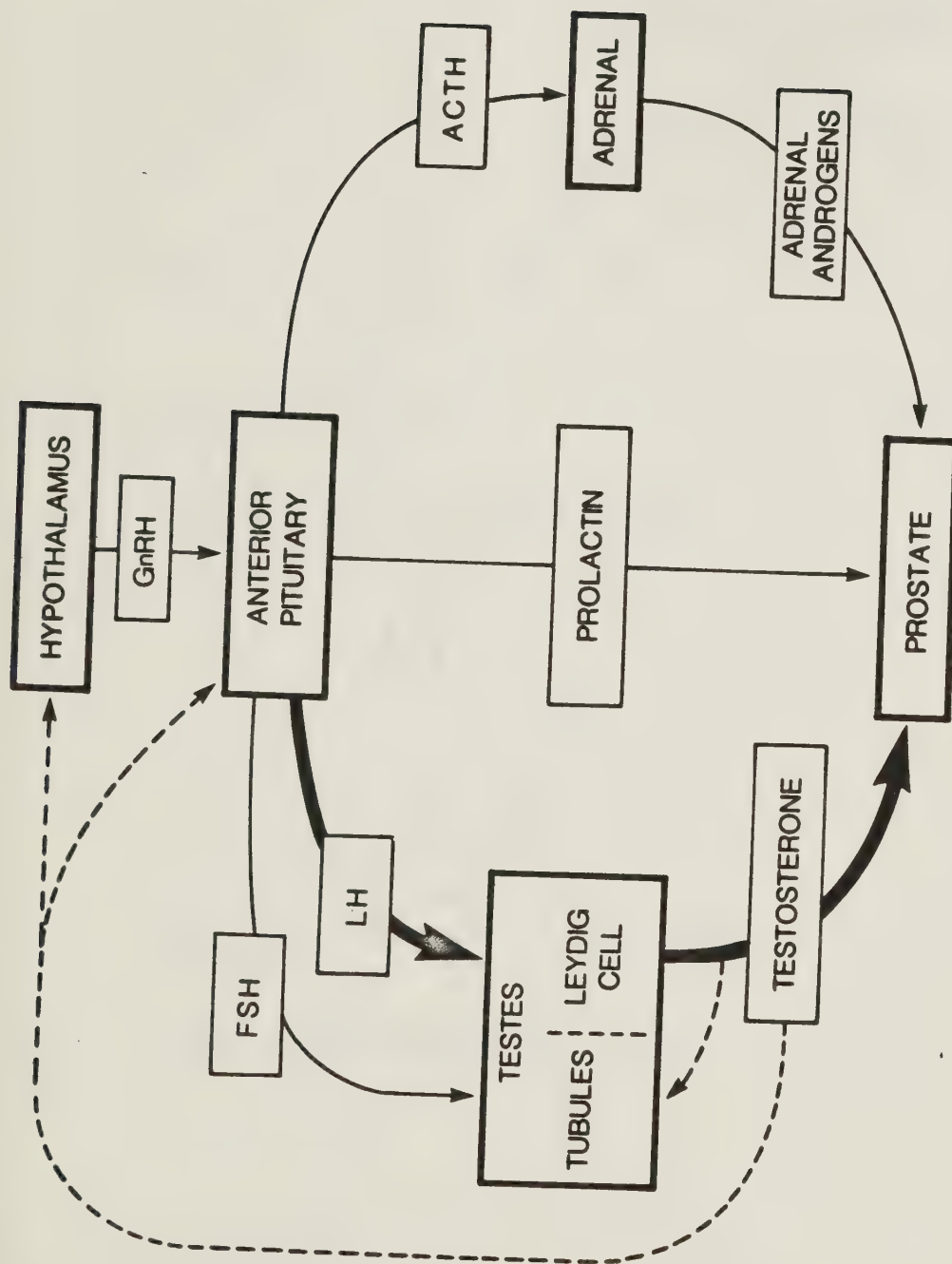


Figure 3. Pituitary-prostate axis: Endocrine control of prostate growth. GnRH = gonadotropin releasing hormone. FSH = follicle stimulating hormone. LH = luteinizing hormone. - - - > = inhibitory feedback.

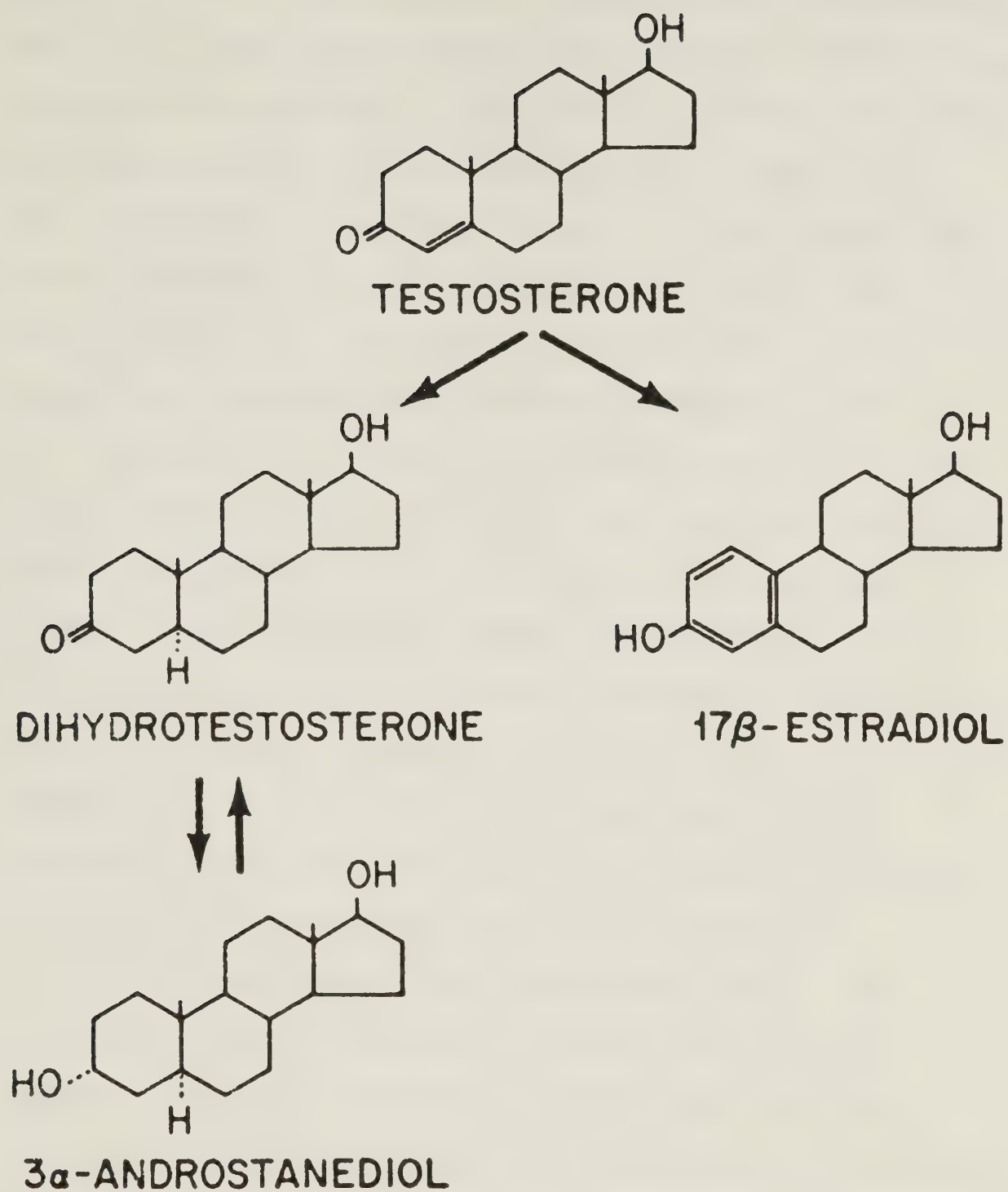


Figure 4. Metabolism of Testosterone.

Isaacs, 1981). Thus the DHT concentration in the serum is very low (50-60 ngm per cent) and it is very tightly bound to the plasma proteins. These facts in general diminish the importance of DHT as a circulating androgen, especially from the point of view of a target organ such as the prostate. In contrast, the DHT formed within the prostatic cells is the major androgen regulating prostatic growth and differentiation (Coffey and Isaacs, 1981).

Peripheral aromatization of testosterone to estradiol accounts for up to 90 per cent of the estrogen in normal human males, while the testes (most likely from the Sertoli cells (Coffey and Isaacs, 1981)) secrete a very small amount of estrogen directly into the blood stream. Thus, in the normal human male, the physiological consequences of testosterone are the result of the combined effects of testosterone itself plus the effects of its 5-Alpha-reduced and estrogen derivatives.

Adrenal androgens play a very small role in the maintenance of the prostate. Adrenalectomy has no apparent effect on prostate size in intact rats, and following castration, the prostate involutes to less than 10 per cent of its original size, despite the presence of normal adrenals (Coffey and Isaacs, 1981). Adrenalectomy and/or hypophysectomy does however bring about some further slight reduction in prostatic size in castrated experimental rats (Arvola, 1961; Tisell, 1970).

With advancing age, the serum concentration of

estrogen in human males increases by approximately 50 per cent (Vermeulen, 1976). This increase is mirrored by an increase in the sex hormone binding globulins (SHBG) and results in the free estrogen level remaining constant (Vermeulen, 1976). However, increase in the sex hormone binding globulin results in more bound testosterone, yielding a fall in the free testosterone level. Therefore there is an alteration in the free testosterone to free estrogen ratio by as much as 40 per cent in favour of estrogen (Vermeulen, 1976).

One of the current theories for the pathogenesis of BPH is that the prostatic cells produce greatly increased amounts of DHT, thereby stimulating increased growth and hypertrophy of the gland (Wilson, 1980). It has been shown that there is a three- to four-fold increase in the DHT concentration in BPH tissue when compared to normal prostatic tissue, (Siiteri and Wilson, 1970; Lloyd et al 1975; Geller et al, 1976; Krieg et al, 1977) and it appears that the increase in DHT is due to increased formation of this metabolite within the cells. In a recent paper, Isaacs and Coffey (1981) have demonstrated an increased prostatic ability for the net formation of DHT in an animal model system (beagle dogs), and this ability for the net formation of DHT was found to be directly related to the incidence of BPH development. In other laboratory experiments using castrated dogs, it has been shown that androgen administration can cause the

development of BPH (Isaacs and Coffey, 1981). If small quantities of estrogen are given at the same time, the incidence of BPH is significantly increased (Isaacs and Coffey, 1981; Jacobi et al, 1978; Walsh and Wilson, 1976) and Moore et al (1979) have shown in dogs that such increased estrogen levels bring about an elevation in the amount of AR within the prostate cells.

Thus with increasing age three phenomena may affect the endocrine status of the prostate: there is an increased production of DHT within the prostate, and, it has been suggested that there may be an increase in the concentration of AR within the prostate cells. Both of these developments would give rise to an increased growth stimulus leading to the development of BPH. Thirdly, the increased estrogen to testosterone ratio in the circulation results in a relatively estrogen-rich milieu, and this is thought to have a role in the pathogenesis of BPH (Mostofi, 1970).

An accepted method to determine whether a tissue is steroid-dependent is to assay for steroid receptors in the tissue. Such assays are being applied to determine hormonal dependence in breast cancer and have been shown to be of value in prognostication and management of this disease (Jensen et al, 1971; Byer et al, 1979; McGuire and Chamness, 1973). Steroid hormone action on target tissues is mediated via specific intracellular proteins known as receptors (Mainwaring and Milroy, 1973). Steroids enter

the cell and bind to the high affinity cytoplasmic steroid-specific receptors to form steroid receptor complexes (see Figure 5). These complexes are transformed (activated) by a poorly defined mechanism to facilitate translocation of the entire complex into the cellular nucleus. Once in the nucleus, the complex binds to certain, as yet unspecified elements of the chromatin (acceptors), and thereby alters gene transcription and the production of messenger RNA (mRNA), to bring about the synthesis of cell-specific proteins, or the modulation of processes which are observed as specific cellular responses to the particular steroid hormone. Therefore, in the cell, steroid receptors may be in the cytoplasm or in the nucleus (Figure 6); the distribution of receptor between these two compartments is dependent on the level of endocrine activity within the host (Barrack and Coffey, 1980).

In studies of nuclear steroid-binding sites, Anderson, Peck and Clark (1973) have shown that only a limited number of the nuclear sites is necessary for estrogenic induction of maximal uterine growth in the rat. These authors have demonstrated that, while uterine cells contained 15-20,000 estrogen receptors (ER) in their cytoplasm, the long term retention of only 1000-3000 estrogen receptor complexes by the nucleus is required for uterine growth (Anderson et al, 1975). Clark and Peck, (1976) and others (Barrack and Coffey, 1980) have observed

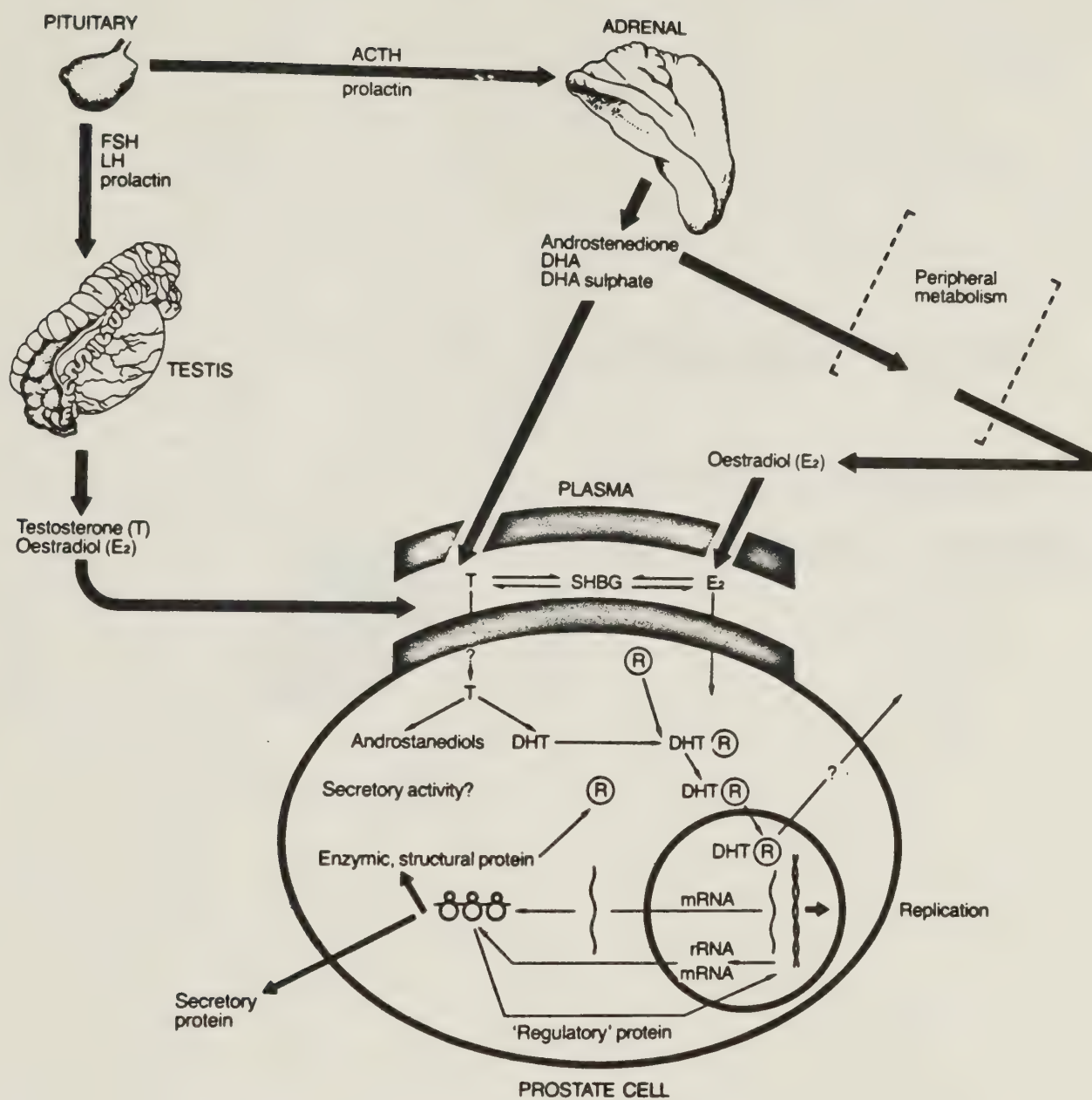


Figure 5. Intracellular action of steroids, mediated by receptor proteins (R).

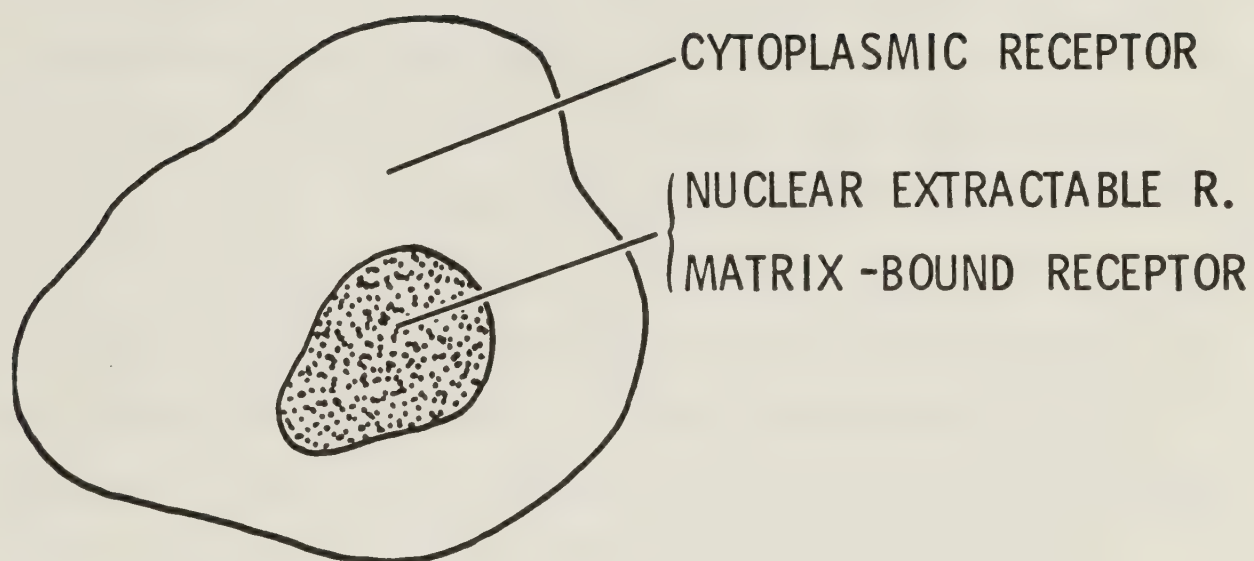


Figure 6. Intracellular location of steroid receptors.

R. = receptor.

that nuclear steroid binding sites exist as two measurable fractions: one fraction is extractable by high ionic strength (0.6M KCl) and the other is resistant to this salt treatment. Long term retention of the steroid receptor complexes in the nucleus is due to binding of these complexes to a limited number of nuclear acceptor sites, equal to the number required for maximal uterine growth (Clark and Peck, 1976). These apparently functional nuclear sites are the ones resistant to extraction with high salt (Clark and Peck, 1976).

Extensive work has been carried out to ascertain the exact site of nuclear action of the steroid hormones. Barrack and Coffey (1980) recently demonstrated the presence of sex steroid hormone binding sites localized in a discrete nuclear subfraction, which they call the nuclear matrix. This nuclear matrix is a chromatin-depleted and salt-insoluble, proteinaceous, intra-nuclear structure. The matrix contains, besides the remnants of an internal protein network, a residual pore complex lamina and residual nucleoli. The functions of the matrix have yet to be elucidated. It has been suggested that nuclear morphology may be related to the presence of the matrix and recently Berezney and Buchholtz (1981) have reported that DNA replication sites are apparently associated with this nuclear matrix. The nuclear matrix has also been implicated in RNA processing (Miller et al, 1978; Herlan et al, 1979), nuclear contractility

(Wunderlich and Herlan, 1977; Wunderlich et al, 1978), control of nuclear membrane fluidity (Wunderlich et al, 1978; Giese and Wunderlich, 1980), steroid hormone binding (Barrack and Coffey, 1980), chemical carcinogenesis (Hemminki and Vainio, 1979), and viral replication (Hodge et al, 1977; Chin and Maizel, 1977). However, the biological significance of matrix-associated steroid binding remains to be delineated.

Recent methodological advances have enhanced the accuracy of steroid receptor assays. Synthetic steroids have improved the reliability of these assays. For example, the ligand methyltrienolone (R1881), a synthetic androgen, binds to androgen receptor (AR) but not to sex hormone binding globulins (Bonne and Raynaud, 1975, 1976; Menon et al, 1978). Also, this ligand is resistant to metabolic conversion and it exchanges with receptor bound endogenous DHT to about 70 per cent during an overnight incubation at 0°C (Bonne and Raynaud, 1975; Menon et al, 1978). The synthetic progestin (R5020) likewise does not bind to transcortin (corticosteroid binding globulin, CBG), but interacts with PgR, even more strongly than progesterone (Bonne and Raynaud, 1975). The non-steroidal estrogen, diethylstilboestrol (DES), binds to ER but not to SHBG, and thus allows for accurate ER assay, when used in competition with [³H]estradiol (Raynaud et al, 1979).

Secondly, the receptor stabilizer, molybdate, has been shown to greatly enhance quantitation of cytosolic

glucocorticoid receptor (GR) and ER in assays (McBlain and Shyamala, 1980; Leach et al, 1979; Mauck et al, 1982) and when applied to AR assays, molybdate has led to significant increases in the levels of cytosolic AR measured (Trachtenberg et al, 1981; Hawkins et al, 1981; Sirett and Grant, 1982).

In early studies of AR in the human prostate, [^3H]-DHT was the ligand used. DHT binds not only to AR, but also to PgR and SHBG, which is present in abundance in the human prostate (Hicks and Walsh, 1979). Consequently, none of the techniques described for detection of AR in human prostate tissue using DHT [density gradient centrifugation (Mainwaring and Milroy, 1973), ammonium sulphate selective precipitation (Geller and Worthman, 1973), agar-gel electrophoresis (Steins et al, 1974), Sephadex chromatography (Hansson et al, 1971; Fraser et al, 1974), and competition by cyproterone acetate (Geller and Cantor, 1975)] was capable of precise measurement of steroid-specific, high affinity binding of androgens to AR. Later studies performed using methyltrienolone (R1881) overcame the problem of ligand binding to SHBG, but R1881 still bound to PgR, thus giving an artificially high estimation of AR levels (Ekman et al, 1979; Asselin et al, 1976; Cowan et al, 1977; Menon et al, 1978). Asselin et al (1979) and others (Hicks and Walsh, 1979; Nozumi et al, 1981; Zava et al, 1979) demonstrated that the addition of an excess of triamcinolone acetonide (TA)

would prevent the R1881 binding to PgR, thus giving a more accurate measurement of AR levels. Hicks and Walsh (1979) also found that an hydroxylapatite assay was more accurate if the protein concentration of the cytosol being assayed was less than one mg/ml, as is frequently the case. The addition of sodium molybdate to the buffers has further improved the accuracy of the assay, and as pointed out above has led to significant increases in the observed levels of AR in cytosolic assays. For the above reasons, until very recently, the results of prostatic AR assays from many centres are not strictly comparable. Now that most laboratories are using R1881, in the presence of an excess of TA, and adding molybdate to the buffers, comparable results for AR in human prostate tissue are being reported.

The detection of ER in human prostatic tissue would suggest a role for estrogen in the growth and differentiation of this tissue. ER has been found in low concentrations in non-hyperplastic human prostate tissue (Ekman et al, 1979; Murphy et al, 1980) and in normal tissue obtained from young renal cadaveric donors (Keen et al, 1982). Also ER has been demonstrated in a proportion of cancer specimens, again in low concentrations (Murphy et al, 1980; Sinha et al, 1973). Despite the work of several groups, ER has yet to be unequivocally demonstrated in human BPH tissue. Some workers have failed to find any evidence of ER in such tissue (Ekman et

al, 1979; Murphy et al, 1980; Keen et al, 1982), while the positive findings of others are open to question because the estrogen binding observed was not shown by various criteria to be typical of binding to high affinity, saturable, hormone-specific ER (Bashirelahi et al, 1976; Hawkins et al, 1975; Pontes et al, 1982; Wagner et al, 1975).

Progesterone receptor (PgR) has been found in some "normal" and most BPH cytosols (Asselin et al, 1979; Ekman et al, 1979; Cowan et al, 1977; Bevins and Bashirelahi, 1980), but the function of this receptor is not understood. Whether the presence of PgR can be taken as an indication of estrogen activity, as in the breast (Horwitz et al, 1978), it is not possible to say. Because of the presence of PgR however, care must be taken when assaying AR, since methyltrienolone binds to PgR when it is present as discussed above.

Nuclear extractable (salt-soluble) prostatic AR has been measured by some groups (Menon et al, 1978; Shain et al, 1978; Shain and Boesel, 1978; Hicks and Walsh, 1979; Lieskovsky and Bruchovsky, 1979; Trachtenberg et al, 1981; Sirett and Grant, 1982) and Murphy et al (1980) have also assayed for the presence of nuclear extractable ER in man. In a recent report, Trachtenberg and Walsh (1982) have reported a correlation between nuclear extractable AR and hormonal responsiveness in 23 men with metastatic prostatic cancer.

Human prostatic nuclear matrices have not been isolated to date, and thus matrix-bound receptors have not been assayed. During the preparation of this manuscript, Barrack et al (1982) have published abstracts on their work assaying non-extractable AR in human prostatic tissue, BPH and cancer. Their assays are carried out on crude nuclear pellets, where no effort has been made to isolate the nuclear matrix from other cellular debris. To date, this is the only report other than that presented herein which describes non-extractable AR in the human prostate.

The purpose of the work presented below was:

1. through the use of an hydroxylapatite assay and cytosolic buffers containing sodium molybdate, cytosolic AR, ER, PgR, and nuclear extractable AR were to be measured to see if any consistent relationship between the three groups of receptors (cytosolic, nuclear extractable and matrix-bound) could be established;
2. a method to isolate nuclear matrices from human prostatic tissue (normal, BPH and malignant) was to be developed;
3. these matrices were to be assayed for androgen receptors (AR).

CHAPTER II

MATERIALS AND METHODS

Tissue Specimens

Benign prostatic hyperplasia (BPH) specimens were obtained at the time of retropubic prostatectomy, and at the time of radical cystoprostatectomy, where nodules of BPH could be easily identified. Histologically proven adenocarcinoma was obtained by transurethral resection of stage C and D prostatic cancers, using a Thompson Cold Punch resectoscope. Specimens taken with a diathermy loop are not suitable, as the induced increase in temperature denatures the receptor protein (Nozumi et al, 1981; Snochowski et al, 1977). Normal prostatic tissue was obtained from renal transplant cadaveric donors between the ages of ten and forty years. In the BPH specimens, care was taken to remove only the hyperplastic nodules for assay, thus obtaining pure hyperplastic tissue. For the collection of malignant tissue, radical prostatectomy specimens are considered unsuitable, as positive identification of the malignant lesion is not always possible (macroscopically), and the possibility of taking benign tissue along with malignant tissue exists.

All specimens were gathered at the time of surgery, and following examination by a surgical pathologist, chopped into small pieces and placed in an ice bucket for

transportation to the laboratory. The tissue was then weighed, frozen by immersion in liquid nitrogen, and stored at -70°C until assayed.

Steroids

Promegestone (R5020) [17-methyl- ^3H] 17,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione (specific activity (s.a.) 87 Ci/mmol), 17β -estradiol, [2,4,6,7- $^3\text{H}(\text{N})$] (s.a. 92 Ci/mmol), R1881 (methyltrienolone, [17-methyl- ^3H] 17-hydroxy-17-methyl-estra-4,9,11-trien-3-one), (s.a. 87 Ci/mmol), [^{14}C] gamma-globulin and [^{14}C] ovalbumin were obtained from New England Nuclear, Boston, Mass., as were unlabelled R5020 and R1881. Unlabelled diethylstilboestrol (DES), triamcinolone acetonide (TA), dexamethasone, estradiol, dihydrotestosterone, progesterone and phenylmethylsulphonylfluoride (PMSF) were obtained from Sigma Chemical Co., St. Louis, Missouri. All other chemicals were of reagent grade, and purchased from Fisher Scientific Co., Quebec. Hydroxylapatite (HAP) was from Bio-Rad Laboratories, Richmond, California. Double distilled water was used to prepare appropriate reagents.

Preparation of Cytosol for the Study of Cytoplasmic

Receptors

Tissue, usually about 1 g, was brought from -70°C to 0°C on ice, and all further steps were carried out at $0-4^{\circ}\text{C}$. The tissue was weighed, chopped with a razor blade and homogenized in 20ml of Tris buffer (10mM Tris, 12mM

monothioglycerol, 10 mM sodium molybdate, 10% (v/v) glycerol, pH 7.4 at 22°C), containing 1mM PMSF, using a Polytron P-10 homogenizer (Brinkmann), set at 4 for 20 seconds (four 5 second bursts with 30 second cooling intervals). The homogenate was centrifuged in a Beckman J2-21 centrifuge at 800 x g for 10 minutes. The supernatant was centrifuged further in a Beckman L2-65B ultra-centrifuge at 200,000 x g for 30 minutes using an SW60 Ti rotor, to yield the supernatant cytosol fraction.

Nuclear Purification and Extraction

Tissue, usually about 1 g, was immersed in liquid nitrogen, and pulverized using a Thermovac pulverizer (Thermovac, Copiague, N.Y.). The powder was suspended in 15 ml of STM/PMSF buffer (0.25M sucrose, 50mM Tris, 5mM $MgSO_4$, pH 7.5 at 22°C) containing 1mM PMSF, and gently homogenized with an all glass Duall homogenizer (Kontes, Vineland, N.J.). This was followed by centrifugation at 800 X g for 10 minutes, and the supernatant discarded. The pellet was resuspended in 15 ml of STM/PMSF, and extracted with 1% Triton X-100 on ice, for 10 minutes. A second centrifugation at 800 X g was done, and the supernatant discarded. The pellet was suspended in 15 ml of STM/PMSF and filtered through a wire mesh (30 mesh) and a third centrifugation at 800 X g followed. The nuclear pellet was then suspended in 25 ml of STM/PMSF and layered on top of 5 ml of 1.8M sucrose for ultracentrifugation at

74,000 X g for 30 minutes, using a Beckman SW28 Rotor in a Beckman L2-65B ultracentrifuge.

The pellet which passed through the sucrose contained purified nuclei, which were extracted twice with 0.6M KCl, and the supernatants saved for steroid binding assays designated as the first KCl extract (see below). The pellet was then incubated with STM buffer containing Deoxyribonuclease (DNase I, Worthington, Millipore Corp., Freehold, N.J., 100 i.u./ml final concentration) on ice for 60 minutes. A final 0.6M KCl extraction was carried out for 15 minutes, followed by centrifugation at 3000 x g for 10 minutes. The supernatant was saved for steroid binding assay, designated as the second KCl extract, and the residual pellet, determined to contain nuclear matrices, was also assayed for steroid binding.

Steroid Binding Assays

In all cases, 200 μ l of cytosol, nuclear extract or nuclear matrices was added to 0.5 ml of hydroxylapatite (HAP) suspension [0.1 g HAP/ml TNP buffer (50 mM Tris, 10 mM sodium phosphate, pH 7.4 at 22°C)] plus 0.5 ml TNP buffer, and shaken for one hour to bind the receptors to the HAP. Following this the HAP suspension was centrifuged at 12,800 X g (Eppendorf) for two minutes and the supernatant discarded. Serial dilutions of steroids prepared as in Table I were added to the residual pellet, the pellet was re-suspended and incubated overnight at

TABLE I. Steroid Dilutions for Saturation Analysis

Solution A				
R1881:	37.5 μ l	1000 nM [3 H]R1881 + 37.5 μ l (1 mM) TA ^{***}	+ 675 μ l buffer	
Estradiol:	85.2 μ l	440 nM [3 H]17 β -estradiol + 665 μ l buffer		
R5020:	85.2 μ l	440 nM [3 H]R5020 + 657 μ l buffer + 8.5 μ l (44 μ M) Dexamethasone		

Solution [3 H] Steroid	Cold Steroid ^{**}	Buffer + 5.68% Ethanol	Volume	Final (nM)	Concentration
1. 264 μ l of A	-	264 μ l buffer	528 μ l	300 μ l	25
2. 228 μ l of 1	-	342 μ l *	570 μ l	"	10
3. 270 μ l of 2	-	270 μ l *	540 μ l	"	5
4. 240 μ l of 3	-	240 μ l *	480 μ l	"	2.5
5. 180 μ l of 4	-	270 μ l *	450 μ l	"	1
6. 150 μ l of 5	-	150 μ l *	300 μ l	"	0.5
7. 216 μ l of A	123 μ l cold ^{**}	93 μ l buffer	432 μ l	"	25
8. 132 μ l of 7	-	198 μ l *	330 μ l	"	10
9. 30 μ l of 8	-	270 μ l *	300 μ l	"	1

* 170.4 μ l Ethanol + 943.7 μ l buffer
** 10 μ l (440 μ M) unlabelled steroid + 490 μ l buffer for Estradiol and R5020
** 4.4 μ l (1 mM) unlabelled R1881 + 495.6 μ l buffer - R1881
*** 50 μ l of 10 mM TA + 450 μ l buffer.

0°C.

A saturation analysis with tritiated ligand in six concentrations (0.1 nM - 5 nM, see Table I) was used to measure total binding and tritiated ligand in the presence of a 100-fold excess of unlabelled steroid was used to measure nonspecific binding (3 concentrations). ER binding was measured using [³H]estradiol-17 β , with or without a 100-fold excess of unlabelled diethylstilbestrol (DES) and PgR was measured using [³H]R5020, with a 10-fold excess of dexamethasone, with or without a 100-fold excess of unlabelled R5020. For AR assays, [³H]R1881 in the presence of 1000-fold excess of TA, with or without a 100-fold excess of unlabelled R1881, was used for the binding assays.

HAP-bound receptors were incubated with steroids for 18-24 hours at 0-4°C. Unbound steroid was removed from the HAP by four washes of 10 mM phosphate buffer. (The HAP pellet was suspended in 1 ml of 10 mM phosphate buffer and shaken for five minutes. This was followed by centrifugation for two minutes at 12,800 x g in an Eppendorf centrifuge. The supernatant was discarded and this process was repeated four times.) The bound radioactivity was eluted from the HAP with 0.5 ml of ethanol. The ethanol extract was added to 10 ml of scintillation fluid (Beckman Ready Solv HP) and the resulting samples counted for radioactivity in a Beckman LS9000 liquid scintillation counter. Specific binding was calculated by subtracting

nonspecific from total binding. The resulting data were analyzed by the method of Scatchard (1949). Specific binding of steroids is expressed in fmols/mg of cytosol protein, and fmols/g of tissue.

Steroid Specificity Determinations

The steroid specificity for the observed ligand binding to ER, PgR, and AR was determined by incubating cytosol with 2 nM of [3 H]estradiol-17 β , [3 H]R5020 plus a 10-fold excess of dexamethasone or [3 H]R1881 plus a 1000-fold excess of TA with or without increasing concentrations of unlabelled steroids. Generally, at least one competitor from each class of sex steroid or glucocorticoid was tested for competition against each tritiated ligand (see Table II).

Sucrose Gradient Centrifugation

Linear 5-20% sucrose gradients were prepared in phosphate buffer (50 mM potassium phosphate, 12 mM monothioglycerol, 10 mM sodium molybdate, 10% glycerol (v/v), pH 7.0 at 22°C) (Dougherty and Toft, 1982) using the ISCO Model 570 Gradient Former. Samples of 200 μ l of labelled cytosol were treated with a dextran-coated charcoal pellet to remove free steroid and an internal [14 C]-labelled standard protein was added prior to layering on the gradients. The gradients were centrifuged in a Beckman L2 65B ultracentrifuge using an SW60 Ti rotor

TABLE II. Steroid Dilutions for Specificity Studies

Incubation No.	Cytosol Volume	$[^3\text{H}]$ Steroid (Final Conc. = (2nM)	Competitor	Competitor Excess
1	200 μl	25 μl	buffer + 5% EtOH in buffer	0 X
2	+	"	(0.04 μM) cold steroid	2
3	"	"	(0.2 μM) cold steroid	10
4	"	"	(1 μM) cold steroid	50
5	"	"	(2 μM) cold steroid	100
6	+	"	(10 μM) cold steroid	500
7	"	"	(20 μM) cold steroid	1000
8	"	"	(2 μM) cold steroid	100 (nonspecific binding)

* 18.2 μ l (440nM) [3 H] steroid + 382 μ l buffer (4.6% EtOH \div 10 \rightarrow 0.46% EtOH into final incubation)

★★ Cold Steroid Dilution: Make up ~ 2 ml of 5mM stock i.e. ~ 2-3 mg steroid/2 ml EtOH depending on the M.W. of the steroid, eg. DES M.W. = 268.3; 5 mM = 2.683 mg/2 ml Actual weighed DES = 3.090 mg + 2.303 ml EtOH

*** For 200 μ M cold steroid: (could use for 10,000-fold excess in some experiments)
80 μ l (5 mM) cold steroid + 1.90 ml buffer + 20 μ l EtOH (5% EtOH \div 10 = 0.5% EtOH into final incubation

For	20 μ M:	16.6 μ l	(200 μ M)	+ 141.9 μ l	buffer	+ 7.5 μ l	EtOH
For 10 μ M:	66.3 μ l	(20 μ M)	+ 63.0 μ l	buffer	+ 3.3 μ l	EtOH	
For 2 μ M:	32.6 μ l	(10 μ M)	+ 123.9 μ l	buffer	+ 6.5 μ l	EtOH	
For 1 μ M:	63.0 μ l	(2 μ M)	+ 59.8 μ l	buffer	+ 3.2 μ l	EtOH	
For 0.2 μ M:	26.0 μ l	(1 μ M)	+ 98.8 μ l	buffer	+ 5.2 μ l	EtOH	

at 240,000 x g for 22 hours. The gradients were fractionated using a Hoefer fractionator and the radioactivity in each fraction determined after the addition of 10 ml Beckman Ready Solv scintillation fluid.

Protein and DNA Assays

Protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad, Richmond, California). Because of the use of molybdate and other possible interfering substances in our buffers, the Bio-Rad protein assay procedure has been validated by comparison with the method of Lowry et al (1951) in this laboratory. DNA was measured by the method of Burton (1956), using calf thymus DNA as standard.

Light and Electron Microscopy

Purified nuclei and nuclear matrices were routinely examined by light microscopy using an Olympus WF10X. Nuclei were stained with methylene blue dye. Samples of purified nuclei and nuclear matrices were fixed at 4°C with 4% gluteraldehyde in cacodylate buffer, before preparation for viewing with the electron microscope. The nuclei were then rinsed in two changes of cacodylate buffer and fixed in 1% OsO₄ (Caulfields' fixative). They were dehydrated through a graded ethanol series and then propylene oxide. The nuclei were embedded in Epon and sectioned on a Reichert OMU2 ultramicrotome. The sections

were stained with uranyl acetate and lead citrate and photographed on a Siemens Elmiskop 1.

CHAPTER III

RESULTS

CYTOSOL

A. Studies of Steroid Receptors in Human Prostatic Cytosol.

1. Androgen Receptor.

a) Saturation analysis of [^3H]R1881 binding in human prostatic tissue:

AR was present in all tissues examined. In normal tissues a mean value of 16 fmols/mg cytosol protein (606 fmols/g tissue) (Table III) with a mean dissociation constant (K_d) of 0.4 nM was observed. In BPH tissue, the mean AR concentration was 22 fmols/mg cytosol protein (595 fmols/g tissue), with a mean K_d of 0.3 nM (Table III). A representative Scatchard plot is shown in Figure 7. AR was present in all 6 cancer specimens at a mean concentration of 21 fmols/mg cytosol protein, (646 fmols/g tissue), but if expressed in fmols/g tissue two of these tissues were relatively AR poor [mean of 349 fmols/g (16 fmols/mg cytosol protein)] compared to the four AR rich tissues [mean of 794 fmols/g tissue (23 fmols/mg cytosol protein)]. The mean K_d for AR in these prostatic cancer tissues was 0.3 nM.

TABLE III. AR Content in Cytosols of BPH, Normal and
Cancerous Human Prostate

Tissue	AR +ve	fmols/mg cytosol protein(a)	fmols/g tissue(a)	K _d (nM) with (Range)
BPH	15/15	22 ± 2.96	595 ± 59	0.3 (0.07-0.57)
Normal	3/3	16 ± 1.86	606 ± 59	0.4 (0.23-0.53)
Cancer	6/6	21 ± 2.44	646 ± 97	0.3 (0.08-0.50)

(a) Mean value of positive samples ± standard error of the mean for positive samples
is indicated

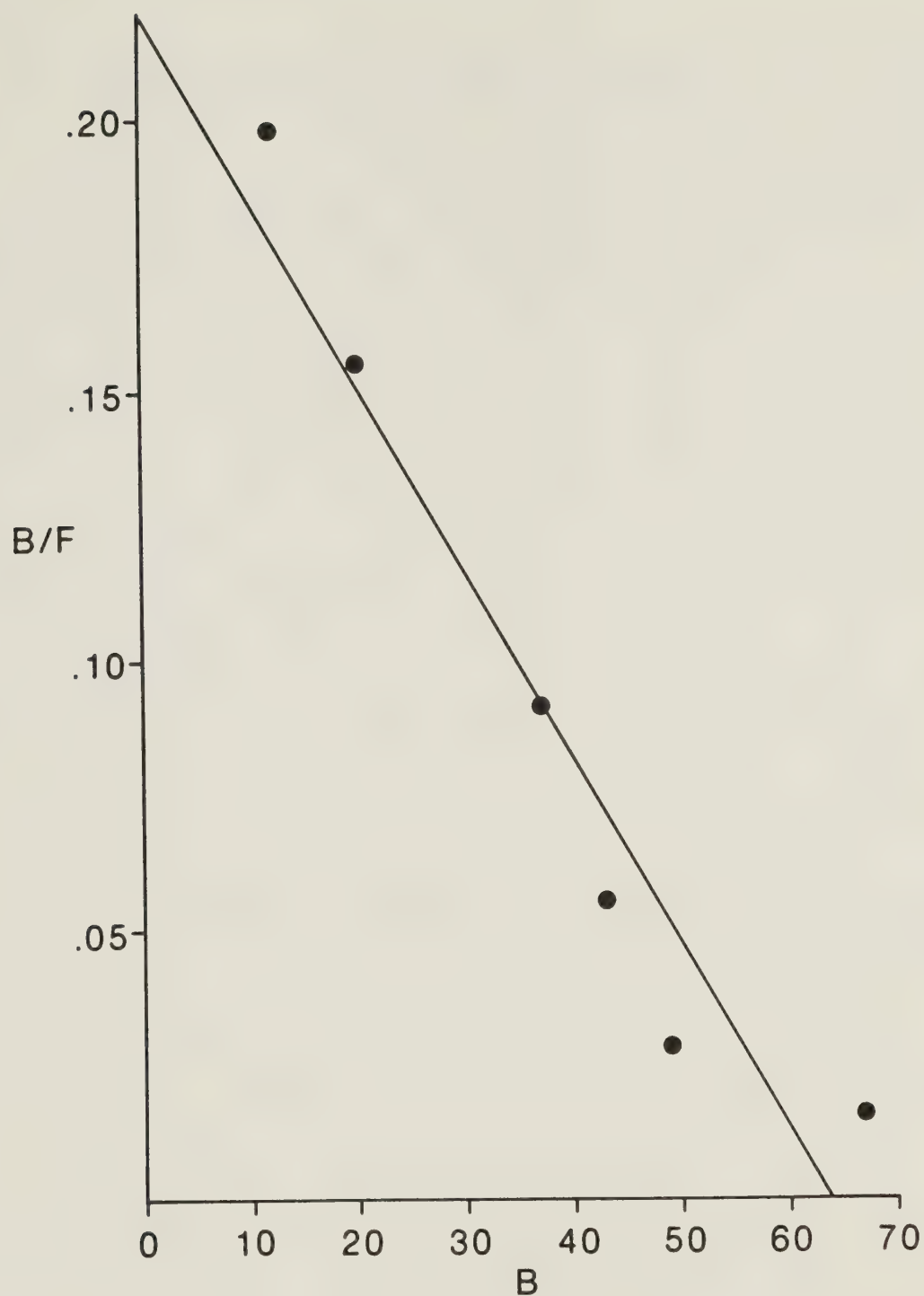


Figure 7. Scatchard plot of cytosol androgen receptor binding in BPH tissue. Maximum binding: 37.4 fmols/mg cytosol protein. $r = -0.97$; $K_d = 0.3\text{nM}$; B = specifically bound steroid (pM); B/F = bound steroid per unit of free steroid.

b) Steroid specificity for binding of [^3H]R1881 in BPH cytosol:

In order to assess whether the observed [^3H] R1881 binding of Figure 7 and Table III was to sites specific for androgenic steroids, steroid competition studies were performed. In Figure 8 are the results of a representative experiment. It can be seen that DHT and R1881 compete for [^3H]R1881 binding. At very high concentrations progesterone begins to compete, as previously reported (Hicks and Walsh, 1979). R5020 also competes in a similar manner to progesterone (data not shown). Estrogen (DES) and a glucocorticoid (dexamethasone) do not compete.

c) Sucrose gradient analysis of [^3H]R1881 binding in BPH:

Further characterization of [^3H]R1881 binding was carried out by sucrose gradient analysis. Cytosolic AR is known to sediment in the 8S region (de Vere White and Olsson, 1981). Figure 9 contains a representative sucrose gradient profile for [^3H]R1881 binding in BPH cytosol and a distinct specific binding moiety is noted in the 8S region. The buffer used in these gradients contains both phosphate and molybdate at pH 7.0, conditions previously found to effectively stabilize other receptors during the long centrifugation time required for this assay (Dougherty and Toft, 1982). This method was applied to three different BPH specimens.

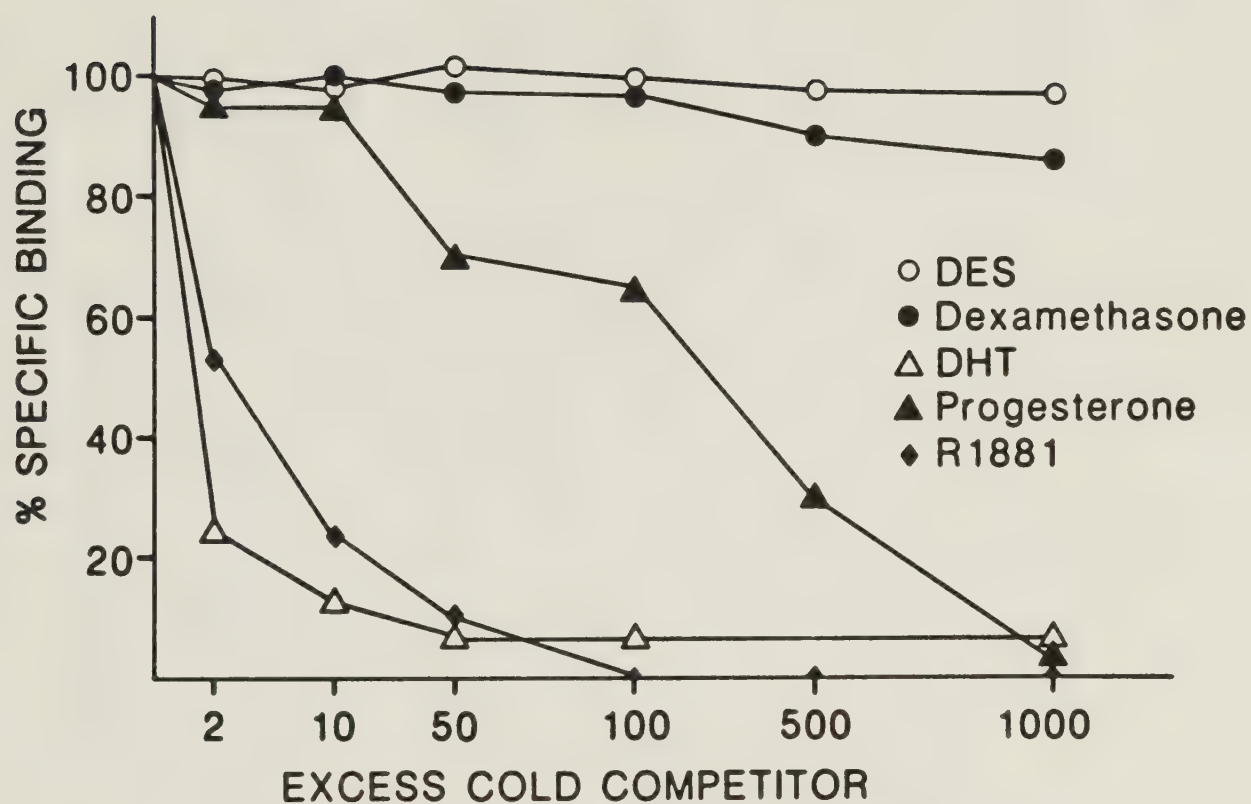


Figure 8. Steroid specificity for binding of $[^3\text{H}]\text{R1881}$ in BPH cytosol. Excess cold competitor units represent the n-fold excess of competing steroid as calculated from Table II.

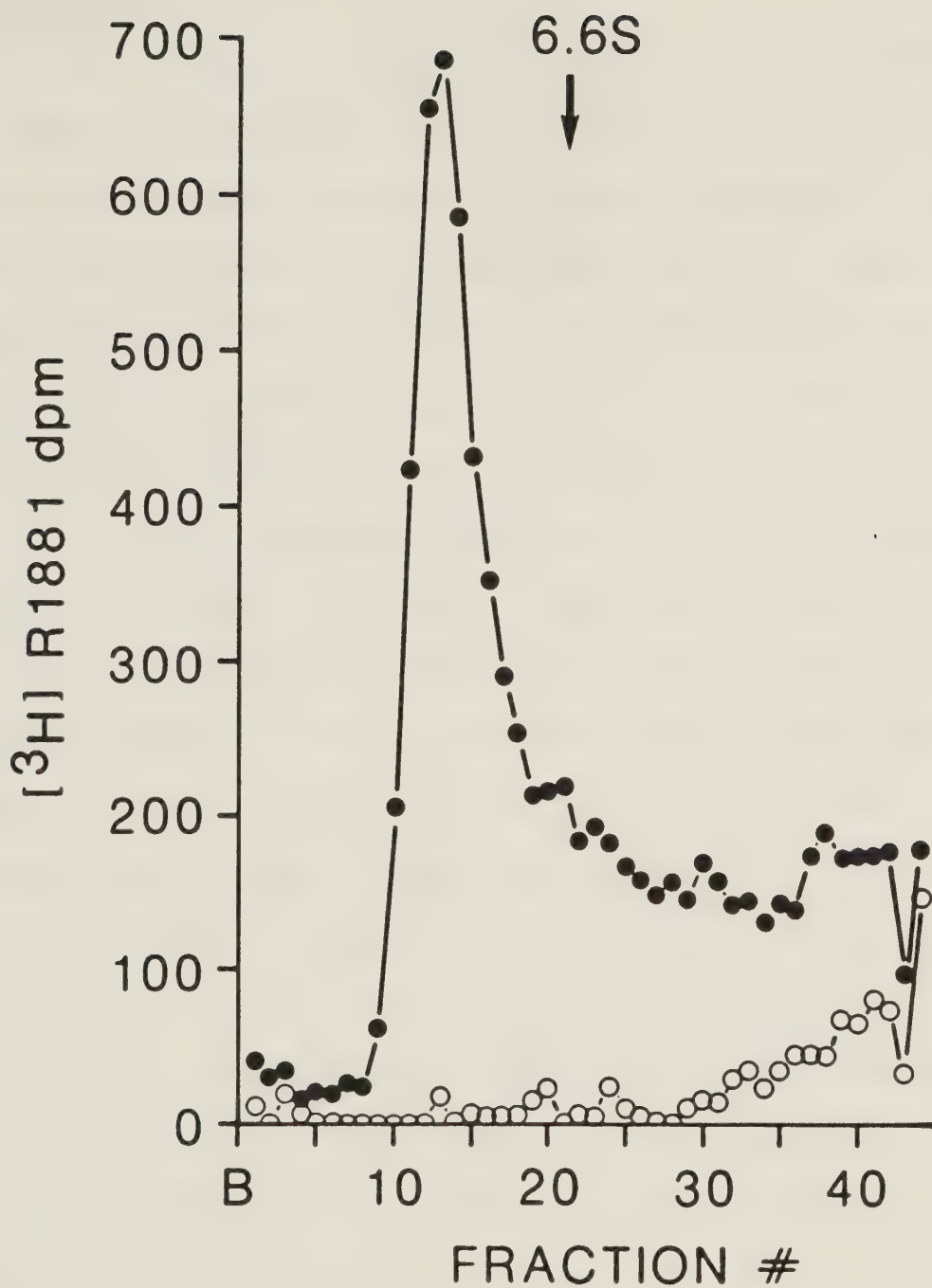


Figure 9. Sucrose gradient analysis of [³H]R1881 binding in BPH cytosol. Total binding (●), non-specific binding (○), and migration of the [¹⁴C] gamma-globulin (arrow with S value).

2. Estrogen Receptor.

a) Saturation analysis of [^3H]estradiol binding in human prostatic tissue:

Shown in Figure 10(a) is a representative plot for the saturation analysis data of [^3H]estradiol binding in human BPH cytosol. Estradiol binding observed in this cytosol was saturable and was displaced by a 100-fold excess of DES. The data were analysed by the method of Scatchard as shown in Figure 10(b) and a single class of high affinity binding sites, typical of ER, was indicated.

Of 15 BPH specimens analysed, 8 exhibited high affinity estradiol binding as indicated in Table IV. The mean ER concentration in the cytosols for which specific estradiol binding could be detected was 10 fmols/mg cytosol protein, (290 fmols/g of tissue) and the dissociation constants (K_d) ranged from 0.01 to 1.2nM.

In a similar fashion, ER was measurable in both normal and cancerous human prostatic tissues as also shown in Table IV. Although the numbers of these two tissue types analysed were small the data seem similar to those for BPH with regard to ER concentration, affinity and the proportion of ER-positive samples.

b) Steroid specificity for the binding of [^3H]estradiol in BPH:

In order to assess whether the observed [^3H]estradiol binding of Figure 10 and Table IV was to sites specific

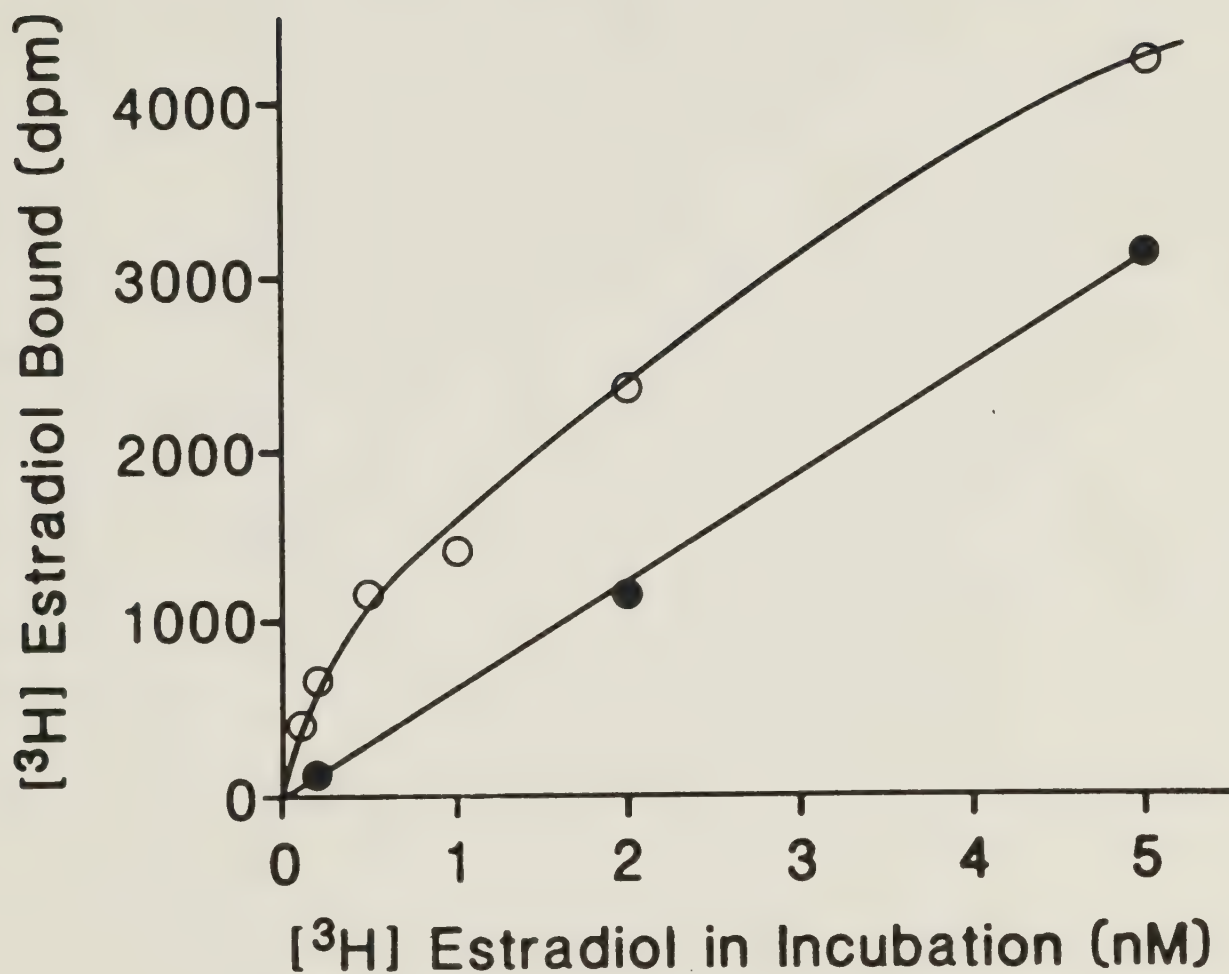


Figure 10(a). Saturation analysis data for [^3H]estradiol binding in BPH cytosol. Total binding (o). Non-specific binding (●).

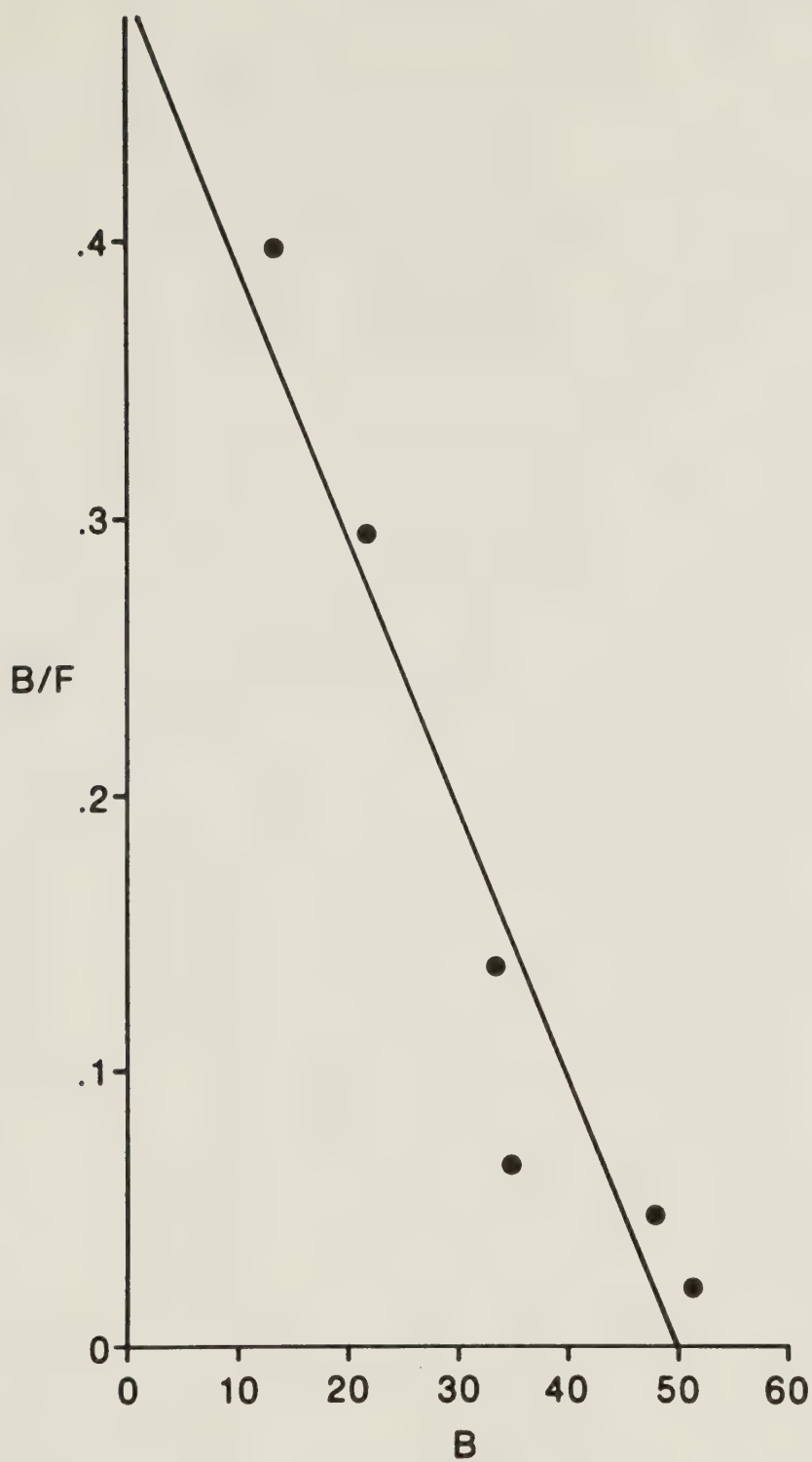


Figure 10(b). Scatchard plot of Estrogen receptor binding in BPH cytosol. Maximum binding: 16 fmols/mg cytosol protein; $r = -0.95$; $K_d = 0.01$ nM; B = specifically bound steroid (pM); B/F = bound steroid per unit of free steroid.

TABLE IV. ER Content in Cytosols of BPH, Normal and
Cancerous Human Prostate

Tissue	ER +ve	fmols/mg cytosol protein(a)	fmols/g tissue(a)	K _d (nM) with (Range)
BPH	8/15	10 ± 2.2	290 ± 55	0.6 (0.01-1.2)
Normal	3/3	9 ± 2.1	335 ± 65	0.3 (0.04-1.0)
Cancer	4/6	13 ± 3.9	440 ± 139	0.8 (0.14-1.2)

(a) Mean value ± standard error of the mean for ER-positive samples is indicated.

for estrogenic steroids, steroid competition studies were performed. In Figure 11 are the results of a representative experiment. It can be seen that only estradiol or DES compete for [^3H]estradiol binding while progesterone, androgens (R1881 or DHT) and a glucocorticoid (dexamethasone) do not compete. This pattern of steroid specificity is consistent with the binding of [^3H]estradiol to ER similar to the ER described for known estrogen target tissues. DES, (a non-steroidal estrogen), unlike estradiol, does not bind to SHBG, but binds to ER in competition with the natural estrogens.

c) Sucrose gradient analysis for binding of [^3H]estradiol in BPH:

Analysis of [^3H]estradiol binding by sucrose gradient is often useful in characterizing the binding. Cytosolic ER from a variety of tissues sediment at 8-9S on sucrose or glycerol gradients (Grody et al, 1982) especially if sodium molybdate is present in the buffering medium (Niu et al, 1981). Figure 12 contains a representative sucrose gradient profile for [^3H]estradiol binding in BPH cytosol and a distinct specific binding moiety is observed in the 8S region of the gradients. This method was applied to five different tissues (four BPH and one normal), in all cases confirming the presence of ER.

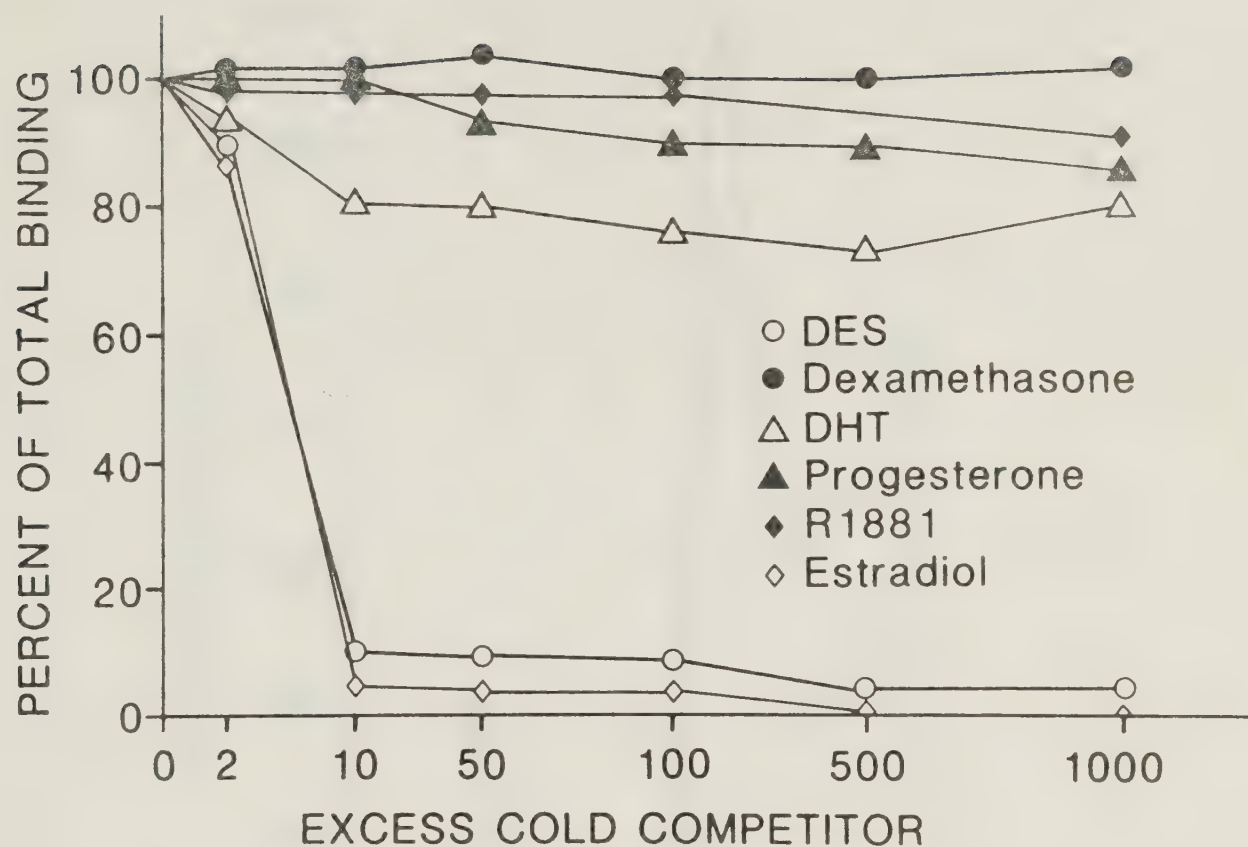


Figure 11. Steroid specificity for binding of [^3H]estradiol in BPH cytosol. Excess cold competitor units represent the n-fold excess of competing steroid as calculated from Table II.

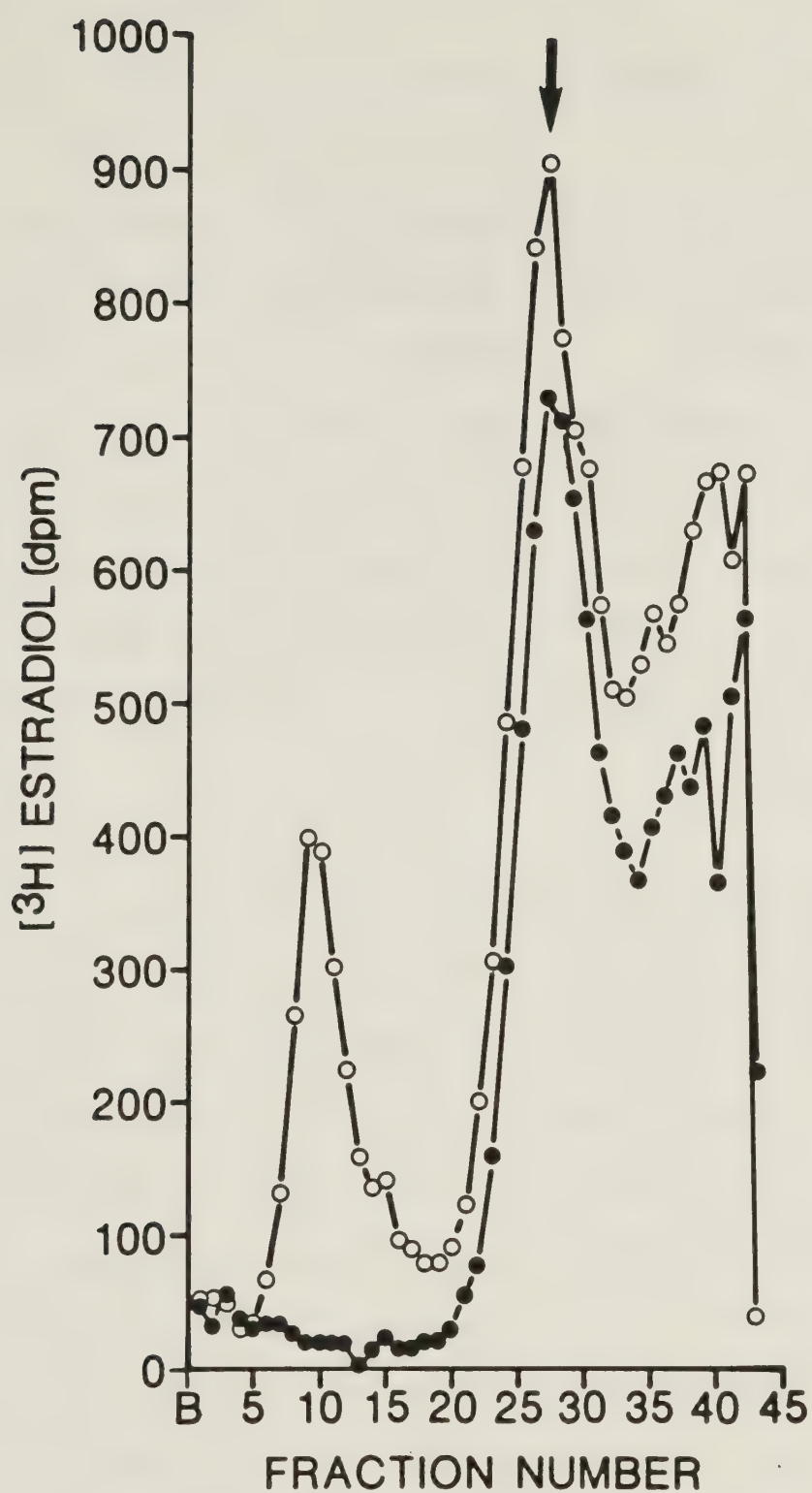


Figure 12. Sucrose gradient analysis for binding of $[^3\text{H}]$ estradiol in BPH cytosol. Total binding (o), non-specific binding (●), and migration of $[^{14}\text{C}]$ ovalbumin (arrow).

3. Progesterin Receptor.

a) Saturation analysis of [^3H]R5020 binding in human prostatic tissue:

PgR was present in all three normal tissues, with a mean value of 21 fmols/mg cytosol protein (745 fmols/g tissue) and a mean K_d 0.1 nM (Table V). Thirteen of 15 BPH specimens had PgR, with a mean concentration of 47 fmols/mg cytosol protein (1446 fmols/g tissue) and a mean K_d of 0.3 nM (Table V). Four of six cancer specimens had PgR, with a mean concentration of 55 fmols/mg cytosol protein (1806 fmols/g tissue), and a mean K_d of 1.3 nM (Table V).

b) Steroid specificity for binding of [^3H]R5020 in BPH:

Figure 13 plots the steroid specificity for R5020. It can be seen that R1881 competes to almost 100 per cent with [^3H]R5020 because it binds to PgR as well as AR (Bonne and Raynaud, 1976). TA also competes with [^3H]R5020, especially when present in 1000-fold excess, when the competition exceeds 80 per cent. This also has been reported previously (Bevins and Bashirelahi, 1980; Kodama et al, 1981). It is for this reason that a 1000-fold excess of cold TA is used when assaying AR (to prevent R1881 binding to PgR). DHT only competes less than 50 per cent as previously reported (Bevins and Bashirelahi, 1980; Kodama et al, 1981; Gustafsson et al, 1978). Estradiol does not compete, as is typical of PgR.

TABLE V. PgR Content in Cytosols of BPH, Normal and
Cancerous Human Prostate

Tissue	PgR +ve	fmols/mg cytosol protein(a)	fmols/g tissue(a)	K _d (nM) with (Range)
BPH	13/15	47 ± 7.62	1446 ± 253.8	0.3 (0.03-0.87)
Normal	3/3	21 ± 4.58	745 ± 49	0.1 (0.01-0.26)
Cancer	4/6	55 ± 17.95	1806 ± 592	1.3 (0.10-2.30)

(a) Mean value of positive samples ± standard error of the mean is indicated.

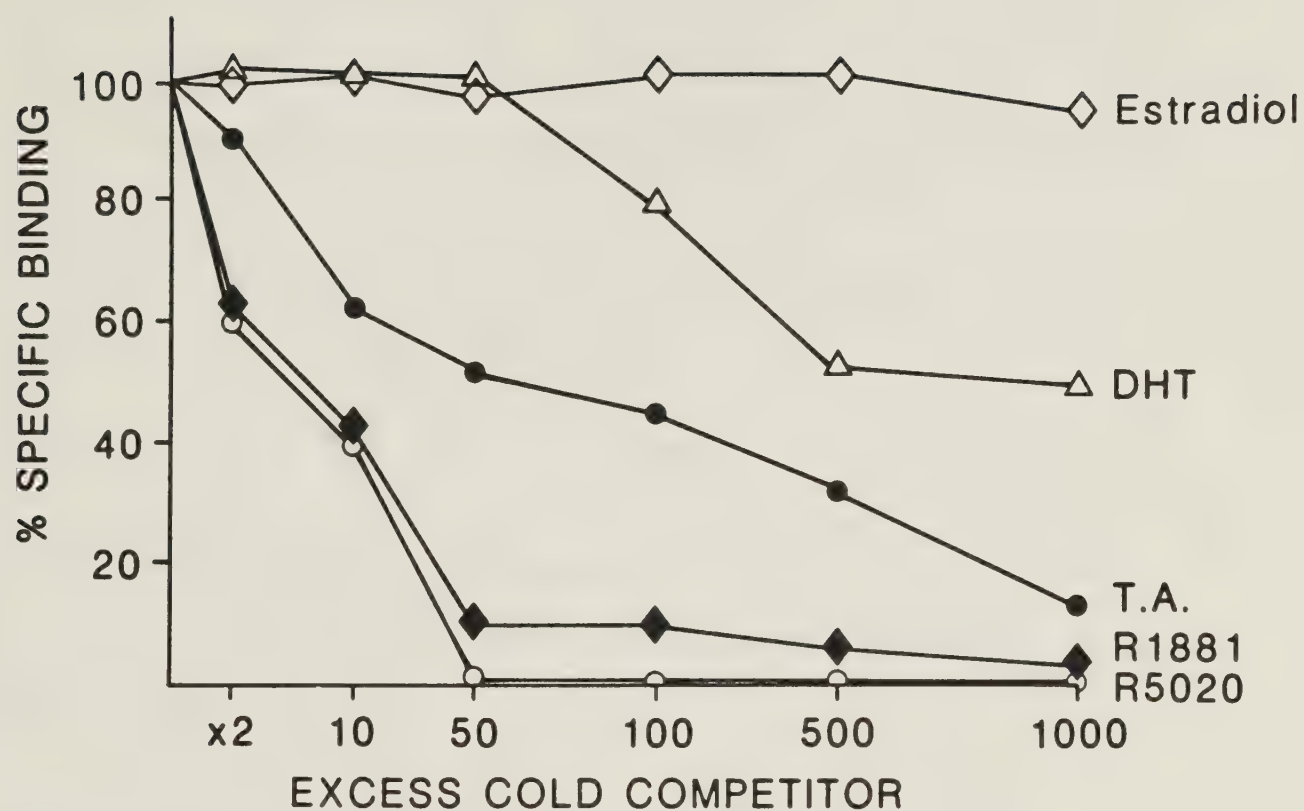


Figure 13. Steroid specificity for binding of [³H]R5020 in BPH cytosol. Excess cold competitor units represent the n-fold excess of competing steroid as calculated from Table II.

c) Sucrose gradient analysis for binding of [^3H]R5020 in BPH:

Similar to the AR and ER experiments presented above, cytosolic PgR from three BPH tissues was characterized by sucrose density analysis. Figure 14 outlines a representative profile and a distinct specific binding moiety is seen in the 8S region, as is typical of cytosolic PgR, which in the native state exists in a 6 - 8S form (Grody et al, 1982).

B. Studies of Nuclear Androgen Receptor in Human BPH:

1. Nuclear Isolation, Purification, and Preparation of Nuclear Matrices:

a) Rat uterine model system:

Nuclear isolation and purification have been described by many workers for various tissues including rat liver (Berezney & Coffey, 1974; Barrack & Coffey, 1980), chicken liver and rat prostate (Barrack & Coffey, 1980), rat uterus (Widnell et al 1967) and human prostate (Lieskovsky & Bruchovsky, 1979). A detailed review of methodology for nuclear isolation in a wide range of tissues is presented by Smuckler et al (1976), Allfrey (1974), Tata (1974), and Spelsberg et al (1974). Since Berezney and Coffey (1974) were among the first to describe the nuclear matrix and its isolation, we decided to follow their methodology initially, as we were also

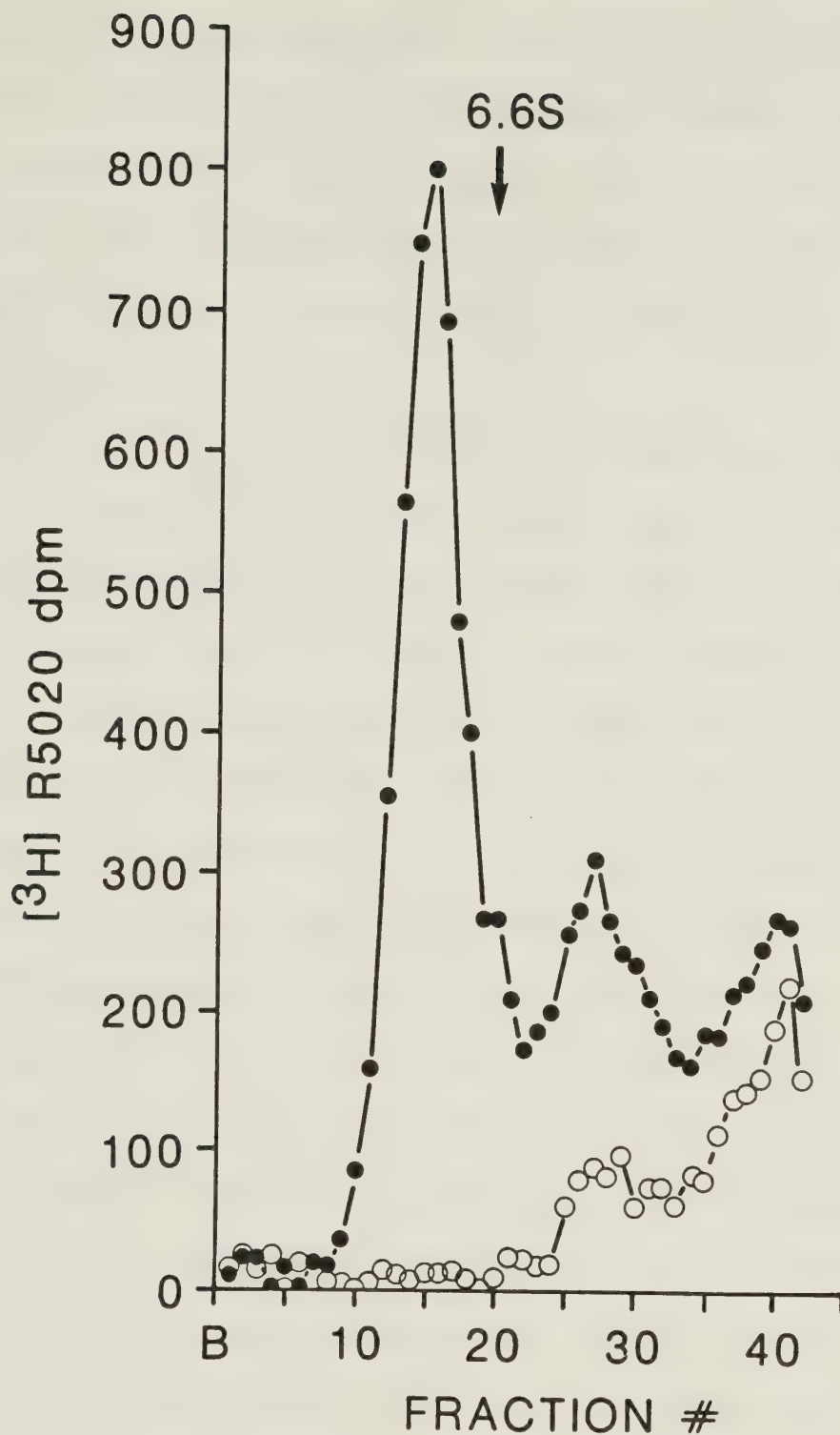


Figure 14. Sucrose gradient analysis for binding of $[^3\text{H}]$ R5020 in BPH cytosol. Total binding (●), non-specific binding (○), and migration of the $[^{14}\text{C}]$ gamma-globulin (arrow with S value).

primarily interested in the nuclear matrix of the prostate. At the beginning of this project, we used rat uterus as a test tissue, since adequate quantities of human prostate were not available, and we wished to establish a satisfactory method for nuclear purification and matrix isolation before using the relatively scarce human prostate tissue.

Using rat uteri and the nuclear isolation methods outlined in the Materials and Methods section above, but with certain modifications as listed below, we attempted to purify nuclei from this animal model system. Rat uterine tissue (1-2 g) was minced, homogenized in 20 ml of STM/PMSF/MTG/MO buffer (see below) with a Polytron homogenizer and centrifuged for 10 minutes at 800 x g. This buffer differed from the buffer described in Materials and Methods since it contained 100 mM monothioglycerol (MTG) and 10 mM sodium molybdate (MO). The residual pellet was resuspended in 10 ml of buffer and filtered through 4 layers of fine cheesecloth instead of the steel mesh. Furthermore, instead of a 1.8 M sucrose purification step, the filtrate was further homogenized and then centrifuged at 800 x g for 10 minutes and the pellet twice more washed in buffer. The remaining nuclei were suspended in 5 ml of STM/PMSF/MO buffer and counted using a Haemocytometer counting chamber and light microscopy.

On light microscopy the nuclei appeared to be well

isolated from other cellular components although a small amount of filamentous debris was present. We had previously seen that the inclusion of 100 mM MTG in the tissue homogenization buffer reduced the debris to a minimum, but as discussed below the MTG may have deleterious effects on assays for nuclear AR. Further attempts toward more pure nuclei using repeated filtrations, HAP or 2.2 M sucrose led to significantly poorer recoveries of nuclei. Typically, the yield of nuclei was 1.7 to 4×10^7 per gram of uterine tissue and on light microscopy the nuclei appeared well isolated.

These isolated rat uterine nuclei were processed to the nuclear matrix stage as outlined in the Materials and Methods section and the nuclear extractable and matrix-bound (non-extractable) receptor assayed. The receptor being assayed was ER, which is present in high concentrations in the rat uterus (Anderson et al, 1973). Uteri from rats pre-treated with a pharmacological dose ($10 \mu\text{g}$) of estradiol (30 minutes before harvesting) were compared with uninjected controls and as can be seen from Table VI pre-treatment with estradiol caused the cytosolic receptor to move into the nucleus and to exist in both salt-extractable and matrix-bound forms.

As discussed below, MTG may cause more extraction of nuclear steroid receptors, but while MTG was being used in these rat uterine experiments it was washed out before nuclear extraction with KCl. Furthermore, a reversal of

TABLE VI. Estrogen receptor distribution in uteri of rats
untreated or treated with estradiol one-half hour
prior to sacrifice

ER Content (Per Cent of Total Measurable ER)		
Material Assayed	Control	Estradiol Treated
Cytosol	56	2
1st Extract	11	27
2nd Extract	10	33
Matrix	23	38

any effects of MTG was probably achieved because the purified nuclei were incubated with 10 mM sodium tetrathionate (NaTT). NaTT has been reported to oxidize sulphhydryl groups to form disulphide bonds (Parker and Allison, 1969; Chung and Folk, 1970; Degani et al, 1974). Kaufmann et al (1981) have shown that this oxidation renders a non-chromatin intranuclear network stable and salt-resistant and we found it enabled us to recover a greater number of intact nuclear matrices. Consequently its use was incorporated into our initial matrix preparations but to our surprise we found that this caused a profound alteration in the distribution of nuclear-extractable versus non-extractable receptor as shown in Table VII. The details of the NaTT effect have not been delineated and are beyond the context of this discussion. For a comparison of results with those of other workers, we wished to make our nuclear-extractable receptor methods the same as those previously employed (Menon et al, 1978; Hicks and Walsh, 1979; Shain and Boesel, 1978; Trachtenberg et al, 1981), so the NaTT treatment was eliminated from the procedure for BPH. Additionally, the value of MTG in purifying nuclei was re-evaluated.

b) Human BPH:

At this point, we felt that the methodology was adequately developed and we were ready to apply it to the

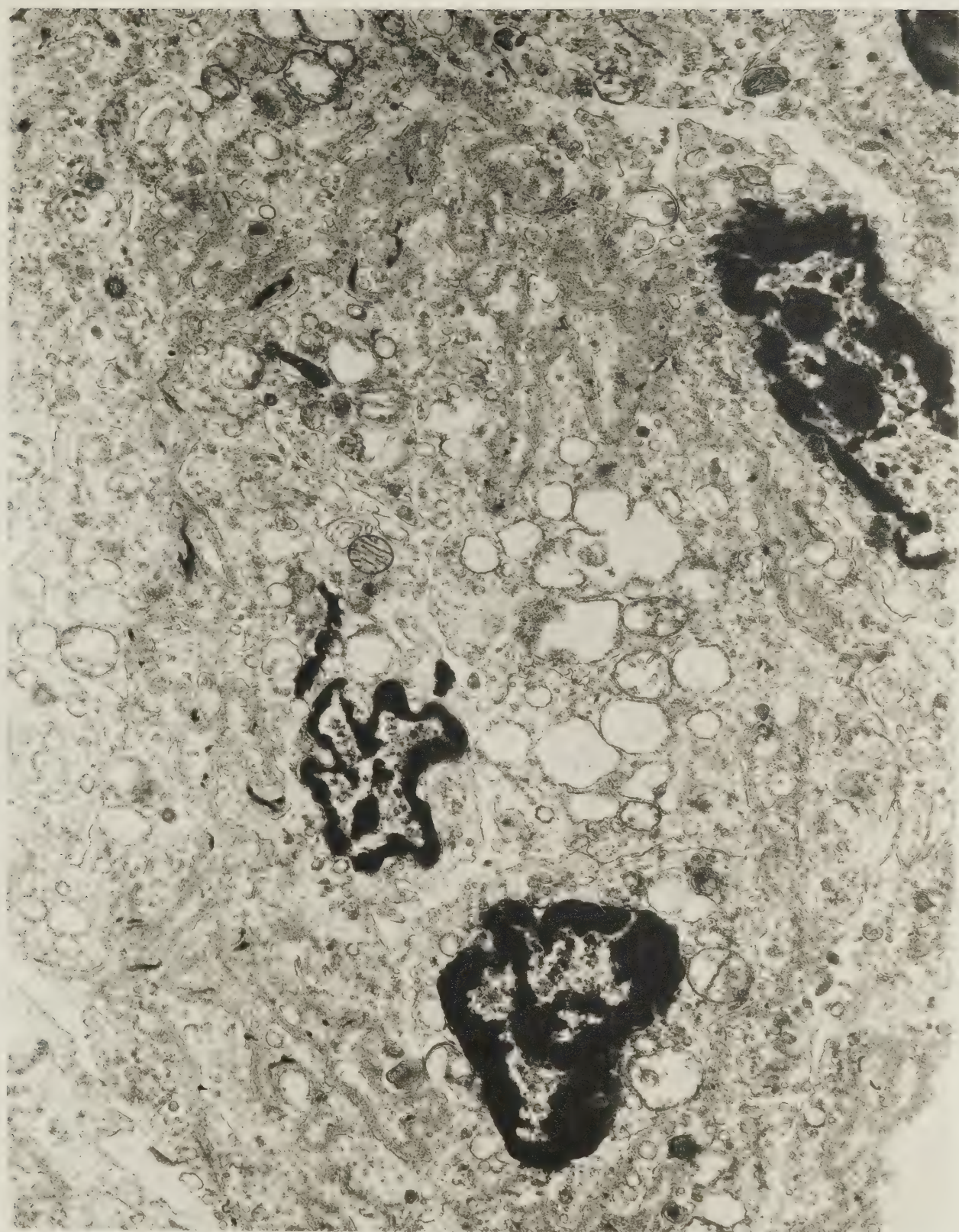
TABLE VII. Distribution of ER in rat uterine nuclei assayed
with or without the use of NaTT in the first KCl
extraction buffer

ER Content (Per Cent of Measurable Nuclear ER)		
Nuclear Material		
Assayed	Control	NaTT
1st Extract	50	20
2nd Extract	20	20
Matrix	30	60

human prostatic tissue. As pointed out above, other research groups were also beginning to evaluate nuclear-extractable androgen receptor in human prostate (Trachtenberg et al, 1981; Sirrett et al, 1982; Menon et al, 1978; Hicks and Walsh, 1979; Shain et al, 1978; Shain and Boesel, 1978; Lieskovsky and Bruchovsky, 1979;) and our methods were similar except that while these workers used crude nuclear pellets, we used relatively well isolated nuclei.

Human prostate tissue (BPH) was processed in the manner described above to yield nuclei, KCl extracts and matrices. The "purified" nuclei were examined under light microscopy. The yield was in the range of $2.2 - 5 \times 10^7$ nuclei/g of tissue, and the nuclei appeared free of cytoplasmic attachments. However, on electron microscopy (EM), it was clear that there was an unacceptable amount of cytoplasmic debris surrounding the nuclei, which had not been apparent on light microscopy (Figure 15). In view of this finding, it was decided to centrifuge the nuclei at $74,000 \times g$ for 30 minutes through a discontinuous sucrose gradient, consisting of 5 ml of 1.8M sucrose layered on top of 5 ml of 2.2M sucrose. It was found that no nuclei passed through the 2.2M sucrose even if the time of centrifugation was increased to 90 minutes as the nuclei were lodging at the interface between 1.8M and 2.2M sucrose. Further sucrose centrifugation therefore used 1.8M sucrose at $74,000 \times g$ for 30 minutes and

Figure 15. Electron-micrograph of impurified nuclei (x 10,000 magnification).



this was found to yield highly purified nuclei as seen on EM (Figure 16).

A further modification was added to the methodology, when it was found that the nuclear yield could be increased from an average of 50 per cent of initial DNA to 70 per cent initial DNA, if the cheesecloth filtration was dispensed with and replaced by simple filtration through wire mesh to remove only the large debris. The low-speed centrifugation and the sucrose centrifugation removed the smaller debris adequately. So, after much trial and error, the nuclear purification methodology as outlined in Materials and Methods was established.

The nuclear matrices were isolated as outlined and their presence confirmed by EM (Figures 17 and 18). Occasional mitochondria were present, but were few in number and some collagen fibres were also identifiable. Some of the matrices were broken to a greater or lesser extent, accounting for the other debris seen in some electron micrographs. This fragility of matrices has also been the experience of other groups working with nuclear matrices (Sufrin et al, 1982) and we have early electron micrographic evidence that NaTT stabilizes these prostatic matrices, as was reported for liver matrices (Kaufmann et al, 1981). Figure 19 (a, b and c) shows nuclear matrices from one tissue, prepared in three different ways: a) No NaTT; b) NaTT added after first KCl extract; and, c) NaTT added before first KCl extract. There were more intact

Figure 16. Electron-micrograph of purified nuclei (x 10,000 magnification).

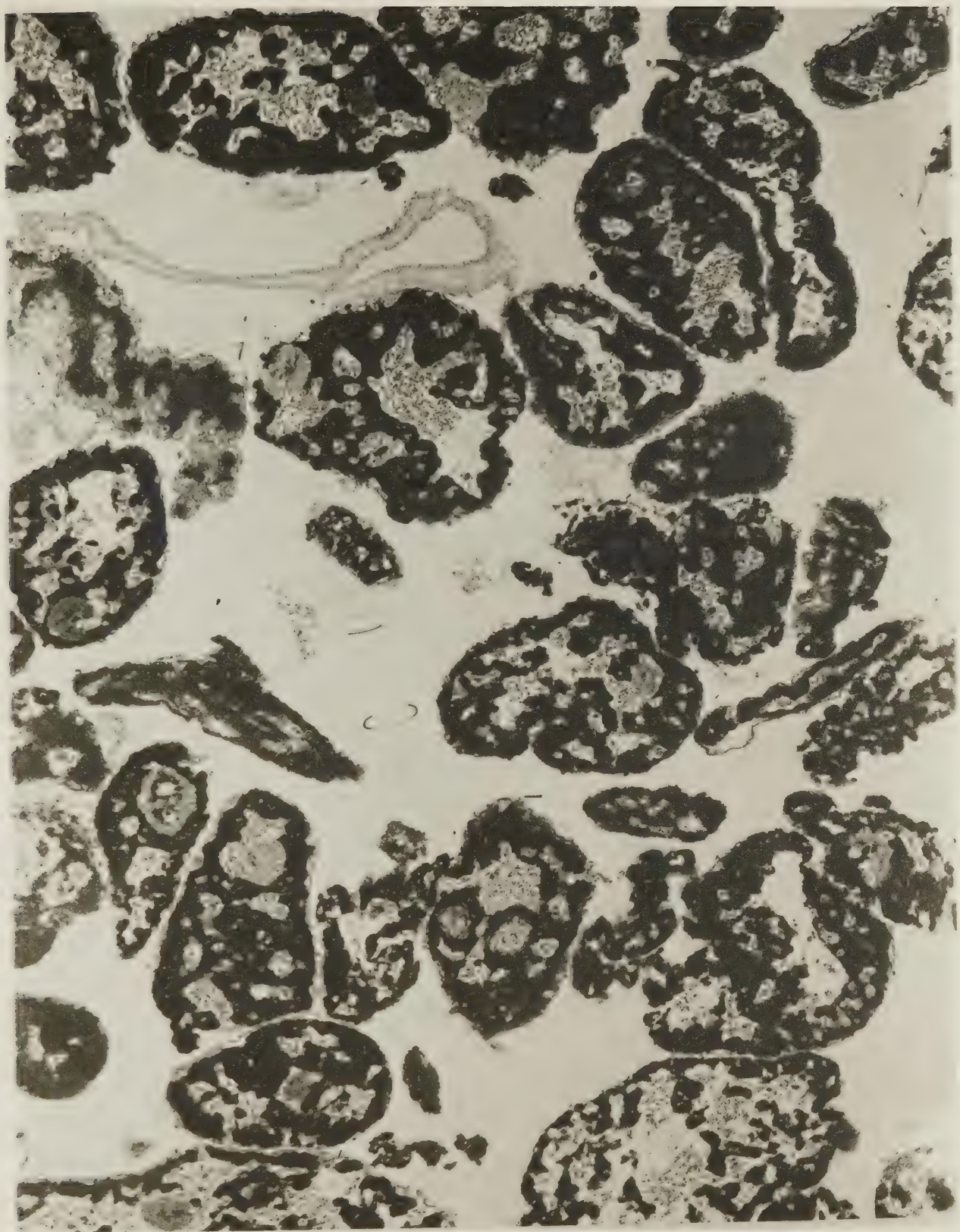


Figure 17. Electron-micrograph of nuclear
matrices (x 14,000 magnification).



Figure 18. Electron-micrograph of nuclear matrix (x 25,000 magnification).

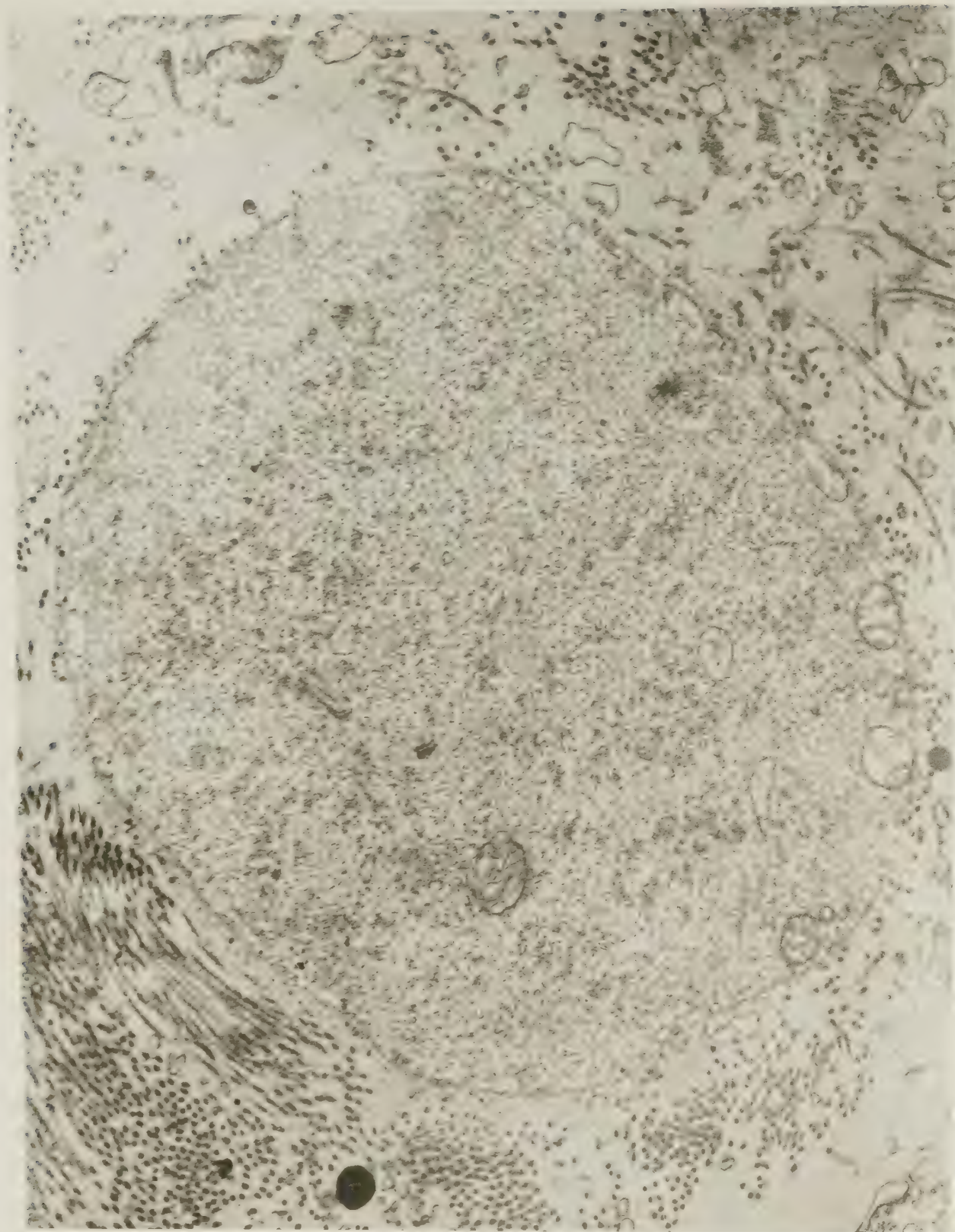


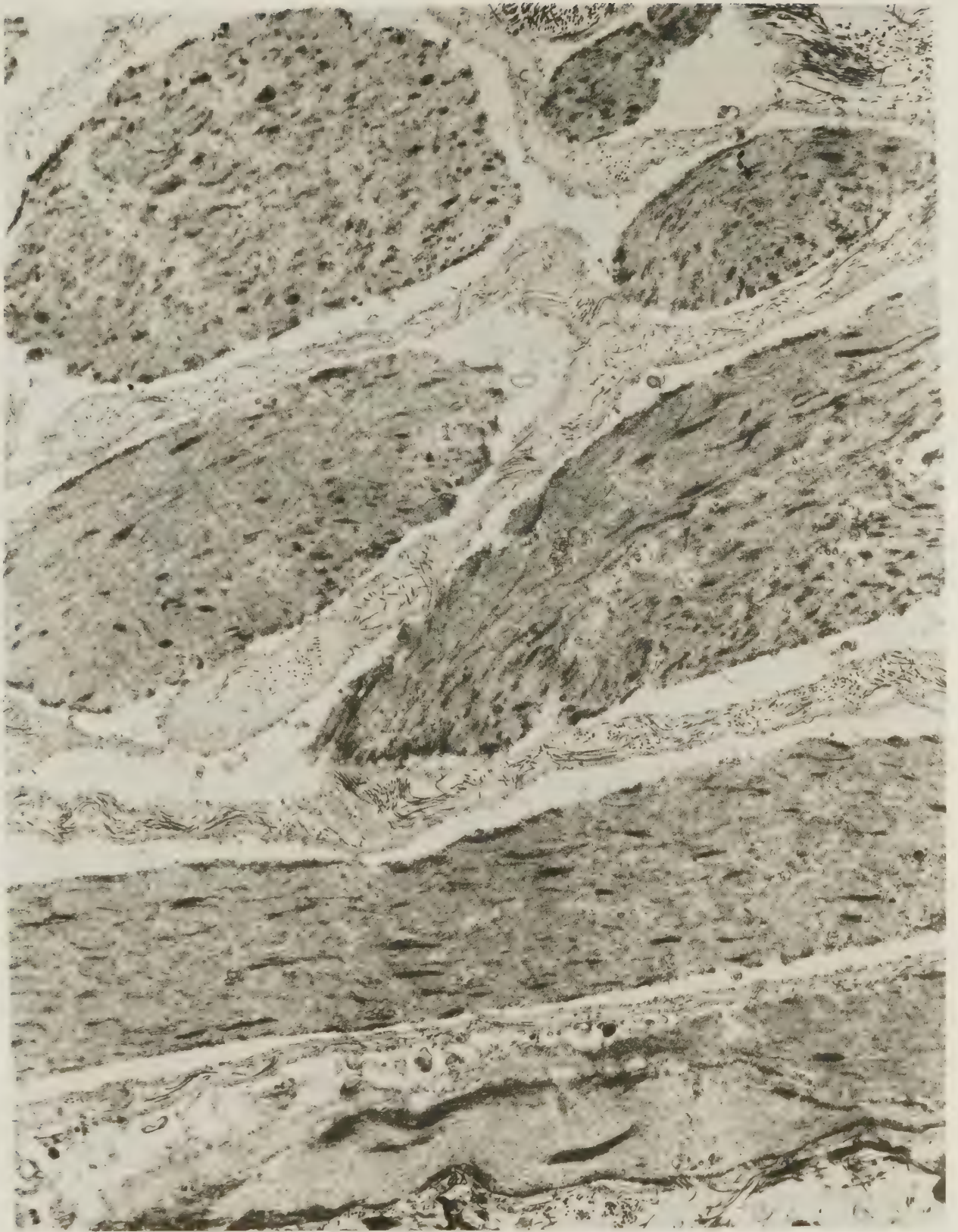
Figure 19 (a). Electron-micrograph of nuclear
matrix (x 25,000 magnification). No NaTT treatment.



Figure 19 (b). Electron-micrograph of nuclear matrix (x 25,000 magnification). NaTT added after the first KCl extraction. Note denser appearance of matrix compared to figure 19(a).



Figure 19 (c). Electron-micrograph of nuclear matrices (x 14,000 magnification). Note dense appearance of matrices, as in figure 19(b). Debris is aggregated into strands. NaTT added prior to the first KCl extraction.



matrices in b) than a), and considerably more in c) than b). The matrices in b) and c) were all more homogenous in appearance, unlike the delicate network of a). In c) the debris is in continuous strands, whereas in a) it is loose and scattered. Thus, NaTT appears to strengthen the nuclear matrices, and cause aggregation of any debris in the preparation.

2. Nuclear Receptor Assays in Human BPH:

a) Preliminary AR measurements:

Nuclear salt-soluble or extractable AR, and salt-insoluble or matrix-bound AR were assayed. Results for the first ten tissues (BPH) were very unsatisfactory, as only low amounts of specific binding were observed; also, the binding was not reproducible, and the Scatchard plots were not linear. AR concentrations were much lower than those previously published (Trachtenberg et al, 1981; Hicks and Walsh, 1979; Sirett and Grant, 1982) and it was suspected that certain methodologic problems existed. In view of this, a visit to the laboratory of Dr. John Trachtenberg at McGill University, Montreal, was arranged to permit a comparison of methods. The work in the Montreal laboratory involved measuring nuclear extractable receptor from crude nuclear pellets so that our nuclear purification scheme as developed above was not in question. The principle difference in methods was that in Montreal the tissue was frozen in liquid nitrogen and then

pulverized in a tissue pulverizer (Thermovac) cooled with liquid nitrogen. The resulting powder was gently homogenized with an all glass Duall Homogenizer (Kontes, Vineland, N.J.) rather than in a homogenizer with blades. The use of liquid nitrogen has the advantage of enabling the tissue to be pulverized into a powder while it is still frozen, whereas the tissue can reach 4°C while being chopped with a razor blade. The rapid freezing and subsequent thawing after liquid nitrogen immersion may be a very effective and gentle way of disrupting cells without disrupting the nuclei.

The Polytron homogenizer is much more vigorous than the Duall glass homogenizer, and even though the homogenized tissue was held in ice while being homogenized using the Polytron, it is possible that there was an increase in the temperature of the tissue during the process. These factors could have contributed to an inactivation or destruction of the nuclear AR, thereby accounting for the unsatisfactory results which we obtained initially. Once the tissue pulverizer and glass homogenizer were used, the nuclear assays gave acceptable values for extractable receptor (see below) and the Scatchard plots were satisfactory. Matrix-bound receptors (or non-extractable receptors) were also detected in large quantities.

b) Saturation analysis of nuclear [^3H]R1881 binding in BPH and normal tissue:

Both extractable and non-extractable (or matrix-bound) receptors were measured by the methods developed as described above. To date, the nuclear extractable receptors reported elsewhere have been extracted from a crude nuclear pellet by one washing in 0.6M KCl; such pellets retained all the nuclear DNA, and accordingly those reports did not take into account any receptors which may only be extracted following digestion of the DNA by DNase I. Barrack et al (1982) however, digest the DNA in their crude nuclear pellets prior to the KCl extraction, by suspending the pellet in DNase I for 30 minutes (25 units/100 μl).

In order to measure those receptors which are freely extractable by KCl and those which are bound to DNA, we carried out KCl extractions both before and after DNA digestion, assaying each extraction separately for AR. Since we have shown that we can successfully isolate the nuclear matrix with a very high degree of purity, we feel justified in calling the non-extractable receptors matrix-bound. This is in clear distinction to the work of Barrack et al (1982) where the non-extractable receptors are assayed from very crude nuclear pellets containing a large quantity of cytoplasmic debris, which in turn could account for some or all of the observed binding in their preparations.

The mean level of receptor in the first KCl extract of BPH tissue was 178 fm/g of tissue (range 0-557) with a mean dissociation constant (K_d) of 3.5 nM (range 0.9 to 7.8) (see Table VIII). Smaller, more consistent levels were found in the second KCl extract, with a mean value of 74 fm/g of tissue (range 0-236), and mean dissociation constant of 3.8 nM (range 0.7 to 10.9) (Table VIII). Significantly higher levels were noted on the nuclear matrices, with a mean value of 574 fmols/g of tissue (range 82-1700), and mean dissociation constant of 2.99 nM (range 0.4 to 5.9). A scatchard plot for one of these matrices is shown in Figure 20.

In the three normal tissues, no receptors were found in the first KCl extract of two of these specimens. In the third specimen, 150 fmols/g were assayed in the first extract. Binding was found in one tissue following the second extract (Table VIII) 247 fmols/g of tissue, with a K_d of 1.7 nM. However, matrix levels in normal tissue (mean 838 fmols/g of tissue, mean K_d of 4.1 nM) were higher than those found in BPH tissue. As the numbers of normal tissue specimens are very small, no statistical analysis was done comparing normal levels with those found in BPH.

As insufficient amounts of malignant tissue were available, no results for nuclear AR in cancer tissue were estimated.

TABLE VIII. Nuclear AR In Human Prostate

Tissue	N	1st Ext. (Mean K_d) ^a	2nd Ext. (Mean K_d)	Matrix (Mean K_d)
BPH	10	178 ± 86 (3.5 nM)	74 ± 23 (3.8 nM)	574 ± 131 (2.99 nM)
Normal	3	150 ^b	247 ^c	838 ± 200 (4.1 nM)

^a Mean ± standard error of the mean for all tissues, expressed in fmols/g of tissue.
^b Level measured in the tissue with extractable AR. Other two tissues negative.
^c Level measured in the one tissue with extractable AR. Other two tissues negative.

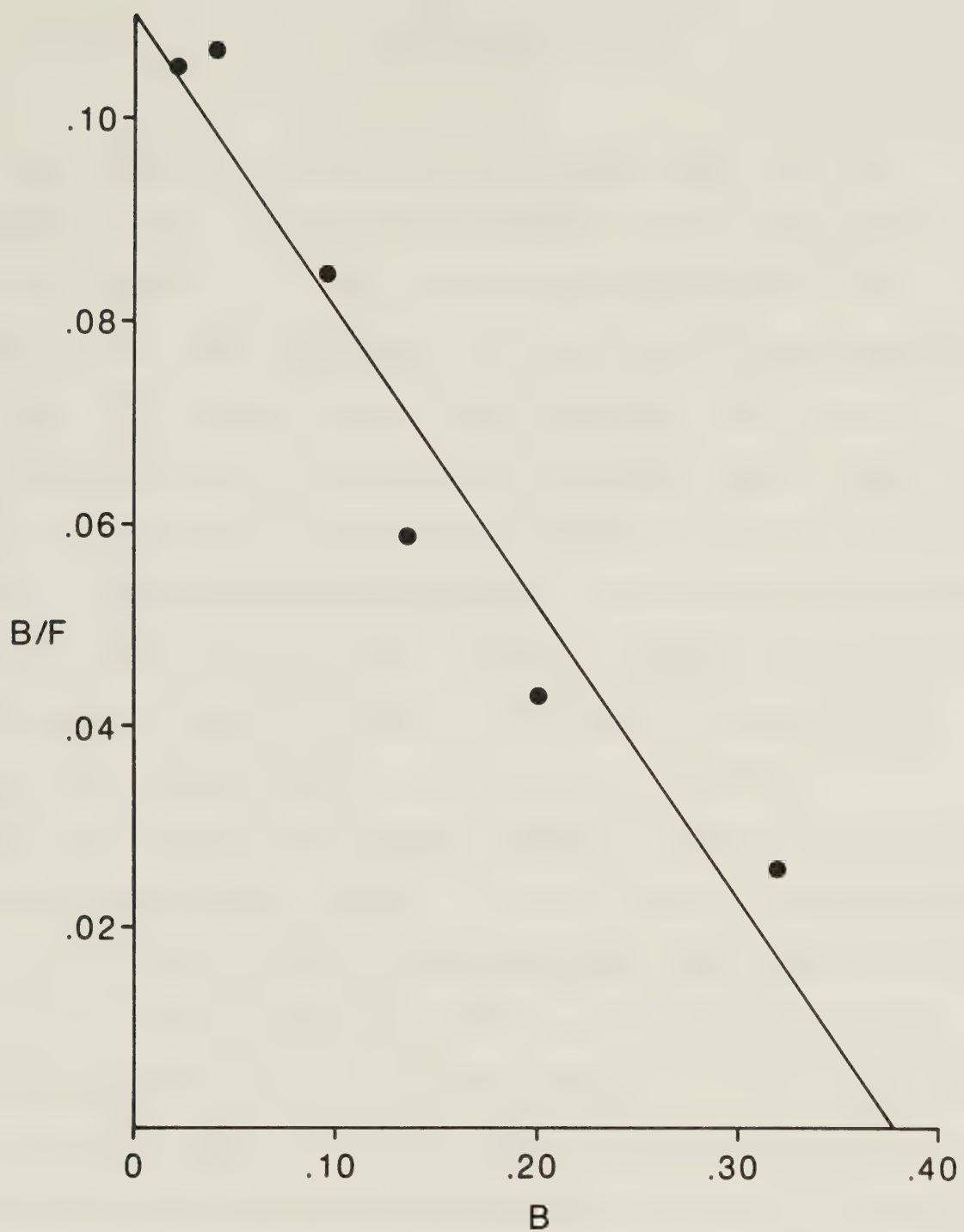


Figure 20. Scatchard plot of matrix-bound androgen receptor. Maximum binding: 647 fmols/g tissue; $r = -0.96$; $K_d = 3.4\text{nM}$. B = specifically bound steroid (pM); B/F = bound steroid per unit of free steroid.

CHAPTER IV

DISCUSSION

The clinical application of cytoplasmic steroid receptor assays in breast cancer has been clearly demonstrated (Byer et al, 1979; McGuire and Chamness, 1973; Jensen et al, 1971) and now is a part of the management of patients with this disease. The value of such assays in the management of patients with prostatic cancer remains to be established. A small early study by Wagner and Schulze (1978) failed to establish any correlation between receptor concentration and clinical response to endocrine management of such patients. Subsequent reports by de Voogt and Dingjan (1978) and Ekman et al (1979) established such a correlation between cytosolic AR and patient response to therapy. A later study (Trachtenberg and Walsh, 1982) failed to substantiate this correlation with cytosolic binding, but did establish a relationship between nuclear salt-extractable AR and clinical response to therapy in 23 cases. This latter study is the only one in which an attempt has been made to establish a correlation among cytoplasmic receptor, nuclear receptor and clinical response.

As yet, no attempt to correlate nuclear matrix receptor levels with clinical responsiveness of a tumor have been reported, since such matrix receptor assays are

only now being established. Since nuclear receptor may be an index of functioning receptor we felt that the nuclear matrix was the most likely area of receptor research to bear fruit from the point of view of clinical application in the future; thus we set out to establish such an assay.

The general method used to measure cytoplasmic receptor is to homogenize a small quantity of tissue in an appropriate buffer. The cytoplasmic receptors are solubilized in the buffer during homogenization, and, following centrifugation of the homogenate, the supernatant, termed the cytosol, is saved for assay. This fraction is then assayed for cytosolic receptors, and the technique is now relatively standardized, as outlined in Materials and Methods.

The cytosolic AR levels reported herein (Table III) are in keeping with published series to date, where sodium molybdate was included in the buffers. When comparing the cytosolic levels presented above with others, one must be careful since sodium molybdate has been shown to cause a two to four fold increase in observed AR concentration in cytosolic assays (Trachtenberg et al, 1981; Hawkins et al, 1981). Also, the use of triamcinolone acetonide (TA) to prevent R1881 binding to PgR, lowers maximum binding levels to as much as one half (Hicks and Walsh, 1979) or even one fifth (Nozumi et al, 1981) of the levels seen in the absence of TA. Interestingly, the effects of

molybdate and TA are counteractive, but both should be used to permit accurate measurement of AR binding sites.

The only studies therefore directly comparable to our own with regard to concentrations of cytosol AR in BPH are those of Trachtenberg et al (1981), Hicks and Walsh (1979), Sirett and Grant (1982) and Keen et al (1982). As can be seen from Table IX, mean values for BPH in our series correlate well with those of Trachtenberg et al (1981) and Hicks and Walsh (1979), but are somewhat lower than those reported by Sirett and Grant (1982). In this latter paper, the number of specimens analysed are not reported and consequently the figures may be skewed due to small numbers. The very high values of Keen et al (1982) just reported, are very difficult to explain (2251 fmols/g protein), even though the methodology was very similar to that used in this study.

Earlier values reported by other authors were measured in the absence of molybdate or TA or both, and as such are difficult to compare; for example, the 35 fmols/mg cytosol protein reported by Ekman et al (1979), the 32 fmols/mg cytosol protein reported by Kodama et al (1981), the 28 fmols/mg cytosol protein reported by Nozumi et al (1981), the 12 fmols/mg cytosol protein reported by Krieg et al (1978), and the 52 fmols/mg cytosol protein reported by Shain et al (1978), are similar to those in Table IX but should not be compared directly. Values for AR in cytosol of normal tissues have been measured by

TABLE IX. Comparison of AR Results (Cytosol) of
Different Studies

Authors	Mean AR Concentration (fmols/mg cytosol protein)
Trachtenberg et al 1981	19 ± 1.04
Hicks and Walsh 1979	26 ± 1.5
Sirett and Grant 1982	38 ± 11
Keen et al 1982	2251 ± 1698 (fmols/g)
Donnelly 1982	22 ± 2.96

Trachtenberg et al (1982) and a mean of 259 fmols/mg DNA (approximately 260 fmols/g of tissue) in 5 cases was reported. This concentration compares with a mean value of 606 fmols/g in the three cases in our study. Again, mean values reported by Keen et al (1982) of 2239 fmols/g in three cases are significantly higher, and difficult to explain.

Observed values of AR in cytosols of cancer specimens in this study were 646 fmols/g of tissue (or 21 fmols/mg cytosol protein), which are comparable with the results of Hawkins et al (1982), (28 fmols/mg cytosol protein) in their series of nine cases. Unfortunately, this group only reported findings for malignant tissue. To date, no other groups have reported cytosol AR levels in malignant tissue measured in the presence of sodium molybdate and TA. It is interesting to note that these levels are almost identical with those found in normal tissue, and not statistically different from those found in BPH (Table III).

What percentage of the total cellular AR the cytoplasmic receptors constitute is difficult to say, as the cytosolic and nuclear assays were not conducted simultaneously in our study. Also, molybdate was used in the cytoplasmic assays, but not in the nuclear ones for the reasons to be discussed below. Molybdate has been shown to cause a loss of nuclear binding (Trachtenberg et al, 1981, see below). The mechanism of this is not under-

stood. Molybdate may behave as a salt, and thus cause lowering of nuclear binding by this action. The numbers of nuclei recovered following purification may influence the nuclear receptor values noted, in that it is possible that one cell type is more numerous following the purification steps, than in the intact tissue. However, if the mean cytosolic level is expressed as a percentage of the total of the mean values for BPH tissue based on tissue weight, it is 42 per cent of the total (Table X). Again, from tissue to tissue, there is considerable variation. Two cancer patients were relatively AR poor (if the levels are expressed in fmols/g of tissue), while the other four patients all had high cytoplasmic levels. This pattern has been previously reported by Ekman et al (1979) who correlated such findings with clinical responsiveness to endocrine therapy. Trachtenberg et al (1982) more recently reported no correlation between cytosolic AR levels and patient response, in 23 cases. Rather, they report a correlation between nuclear-extractable AR levels and patient response to therapy, and patient survival. We have not carried out any clinical studies but these may be initiated in the near future.

Estrogens have been proposed as a major etiological factor in the pathogenesis of BPH in man, (Mostofi, 1970) and this proposal has been supported by studies of canine models, (Walsh and Wilson, 1976; Jacobi et al, 1978; Isaacs and Coffey, 1981). Estrogens have been shown to

TABLE X. Cellular AR Distribution in BPH Tissues
Assayed in this Study

AR Concentration		
Cytosol	Nuclear Extractable	Matrix-bound
595 fmols/g tissue (42%)*	252 fmols/g tissue (18%)*	574 fmols/g tissue (40%)*

* Calculated from mean values for each cellular location
expressed in fmols/gm of tissue.

act synergistically with androgens in the induction of canine BPH (Walsh and Wilson, 1976; Jacobi et al, 1978; Isaacs and Coffey, 1981) and as well it has been observed that canine prostatic androgen receptor concentrations are elevated by estrogen treatment (Moore et al, 1979). If estrogens act directly on the human prostate, ER would be expected to be present in prostatic tissue. Not surprisingly, ER has been found in both canine and rat prostates (Chaisiri et al, 1978; Armstrong and Bashirelahi, 1978; Jung-Testas et al, 1981).

The detection of ER in human prostatic tissue would support a role for estrogen in the growth and differentiation of this tissue. ER has been found in normal tissue in our three cases, and these findings are borne out by Keen et al (1982) in tissue obtained from renal cadaveric donors, and by Murphy et al (1980) and Ekman et al (1979) in non-hyperplastic tissue. Low concentrations of ER have also been demonstrated in a proportion of cancer specimens (Murphy et al, 1980; Pontes et al, 1982; Elliott et al, 1981; Krieg et al, 1978). Our series has ER in four of six cancer tissues and the observed higher concentrations compared to previous reports (Murphy et al, 1980; Elliott et al, 1981) are most likely attributable to our use of sodium molybdate in our homogenization buffer.

The fact that PgR was present in both normal tissue and all four cancer tissues that were ER positive, supports the suggestion that estrogen has a role in

prostatic metabolism, since it is known that estrogens enhance the production of PgR in a variety of target tissues (Horwitz et al, 1978; Milgrom et al, 1973). ER in malignant tissue adds credence to the view that exogenous estrogens may in some cases have a direct effect on prostatic cancer tissue. Further support for this idea was recently presented by Hudson (1981) who demonstrated that upon addition of estradiol to the medium of human prostatic cancer tissue cultures, there was a fall of acid phosphatase production in 10 of 46 specimens. Somewhat more disconcerting was the fact that the acid phosphatase levels increased in 8 of 46, suggesting that estradiol in some way increased the metabolic activity of the cells. Bearing these facts in mind, it is possible that, in patients whose cancer tissue is ER- and PgR-positive, a progestational antiandrogen (such as cyproterone acetate) may be a more suitable endocrine agent than estrogen. Thus, these assays may have a role to play in the clinical management of patients with prostatic cancer, by selecting out that particular group of patients, that respond unfavorably to estrogen.

The inability to unequivocally demonstrate ER in all previous studies of human BPH was disappointing and somewhat surprising. Many workers simply failed to find any evidence of ER in such tissue (Murphy et al, 1980; Ekman et al, 1979; Keen et al, 1982; Shain et al, 1978), while the positive findings of others are open to question,

because the estrogen binding observed was not shown by various criteria to be typical of binding to high affinity, saturable, hormone-specific ER. These earlier reports of ER in BPH are considered below.

The negative results of Ekman et al (1979), Murphy et al (1980) and Shain et al (1978) may be explained by three factors. None of these groups used the receptor stabilizer, sodium molybdate, and these groups also failed to use a proteolytic enzyme inhibitor, such as PMSF, in their preparation, thus allowing endogenous proteases to continue to act. Furthermore, in all series, dextran-coated charcoal (DCC) assays were used, which may, as a result of receptor protein adsorption to the DCC, have caused loss of binding if receptor concentrations were low. The use of an hydroxylapatite assay would overcome the latter two problems, and the use of molybdate would further enhance the probability of detection of ER, if present, as shown by the results in this report. In the light of the above, the negative findings of Keen et al (1982) in 10 BPH specimens are difficult to explain. They used molybdate and also an hydroxylapatite assay. While they do not appear to have used PMSF, the use of hydroxylapatite should have helped to overcome any problem with proteolysis.

The positive findings of Bashirelahi et al (1976) for ER in BPH tissue are probably due to binding to sex-hormone binding globulin (SHBG), as inappropriate ligand

systems were used (labelled and unlabelled estradiol, both of which bind to SHBG and to receptor). A later publication by the same group (Bashirelahi et al, 1979) again reported ER in human BPH tissue, using the standard ligand system (labelled estradiol, unlabelled DES). Unfortunately, only single point analysis was performed, rather than saturation analysis. Single point assays can often be misleading because they only measure estradiol binding at one concentration and as such are not acceptable as unequivocal evidence of a receptor protein because no estimate of affinity is calculated. As Murphy et al (1980) point out, in their study, 10 of 12 cytosols that were negative on saturation analysis would have been positive if only a single point assay was performed at 2 nM of estradiol.

Using agar gel electrophoresis, Hawkins et al (1975), de Voogt and Dingjan (1978), and Wagner et al (1975) reported estradiol binding in human BPH tissue. However, as Hawkins et al (1976) point out, this estrogen binding protein differs from the typical estrogen receptor in its failure to precipitate with protamine, its instability in response to dextran-coated charcoal extraction, and the fact that its sedimentation co-efficient is 4S instead of the 8S seen for more typical cytosolic estrogen receptor. This latter discrepancy may be explained by the absence of molybdate, in which case, the receptor-steroid complex may be activated or transformed into its nuclear

configuration during the preparation process, thus changing from 8S to 4S (Grody et al, 1982). However, it may also mean that what was being measured was in fact a non-specific steroid binding protein such as SHBG. The steroid specificity studies carried out by de Voogt and Dingjan (1978) suggest that the latter is the case, since only naturally occurring estrogens competed with [^3H]17 β -estradiol, whereas DES and Tamoxifen did not.

The major drawback of the agar gel electrophoresis method used by all these groups, is that one cannot calculate the binding affinity or dissociation constant for the binding protein in order to help differentiate between low affinity non-specific binding, and high affinity receptor binding.

Pontes et al (1982) recently reported finding ER in human BPH cytosols, using the three methods of DCC assay, sucrose gradient analysis and high-pressure liquid chromatography (HPLC). This group also used sodium molybdate in their buffer systems. Using HPLC they found a mean level of 20 fmols/mg cytosol protein in five of six tissues examined; binding levels were lower by the other two assay methods. However, the binding affinity is very low at 7.4 nM (only one K_d being reported), no steroid specificity studies were carried out, and the sedimentation co-efficient of the estrogen binding protein was not reported despite the fact that sucrose gradient analysis was performed. The omission of these important items of

information call into question the significance of the findings.

During the preparation of this manuscript, Auf and Ghanadian (1982) report finding ER in the cytosol of 18 of 19 BPH tissues assayed. They used sodium molybdate in their buffers and report mean levels almost identical to those reported herein. The steroid specificity is also similar to that reported herein. However, the dissociation constants reported are very high for ER (mean 2.9 nM), with a very wide range (S.D. \pm 2.6 nM), a finding which would lead many workers in the field to suggest that the binding may not in fact be to a typical estrogen receptor. Furthermore, the sedimentation co-efficient of 4S for the cytosolic receptor is not what one expects for a typical cytosolic ER. This may be explained by the fact that these workers did not use a proteolytic enzyme inhibitor (such as PMSF) in their buffers and the smaller sedimentation co-efficient may be the result of endogenous proteases cleaving the receptor into smaller units.

As pointed out in our results, seven of the eight ER-positive BPH tissues also had high levels of PgR. Six of the seven ER-negative tissues also had PgR in large quantities. If the presence of PgR in the prostate is an index of estrogen activity, as is the case in human breast cancer (Horwitz et al, 1978; Milgrom et al, 1973) this finding lends further support to the proposal that estrogen has a role in the pathogenesis of BPH in man.

Since a major site of action of steroid hormones is in the cell nucleus, one of the objectives in this project was to isolate the cell nucleus as free of cytoplasmic contamination as possible. Once this was achieved, the nuclear matrices could be isolated and assayed for steroid binding sites. The use of pure nuclei would give considerable confidence that any steroid binding detected was binding to matrix components, and not binding to any residual cytoplasmic components. Tissue homogenization is a universally accepted way of breaking up cells without destroying the nuclei. This achieved, cytoplasmic debris has to be removed, and the nuclei rendered as clean as possible. Since the nuclei are the largest and most dense elements remaining after homogenization, they will sediment most rapidly on slow centrifugation, leaving the smaller, less dense cytoplasmic debris suspended in the supernatant. The greater the number of washings of the pellet therefore, the more debris one removes.

Initially it was intended to carry out both cytosolic and nuclear assays on the same piece of tissue, but this was not possible, for several reasons. First, it was not possible for one person to perform cytosolic AR, ER and PgR assays and at the same time isolate pure nuclei, perform the nuclear extractions, and then assay the nuclear receptors. Second, it was found that the nuclear assay conditions were not optimal if the homogenization

was performed by the Polytron homogenizer, whereas the cytosol assays worked well using this simple homogenization method. Furthermore, the use of sodium molybdate (while being an important stabilizer for cytosolic assays) has been shown to interfere with observed nuclear binding levels (Trachtenberg et al, 1981). Finally, Walsh (1982) has reported that the use of the reducing agent DTT (an equivalent of monothioglycerol in its effect) altered the ratio of extractable to non-extractable receptors in his crude nuclear preparations, and that in his laboratory, such agents were not used in nuclear assays. We, likewise, excluded reducing agents from the buffers used in nuclear assays.

For the above reasons, cytosolic and nuclear assays were carried out on separate tissue specimens at different times and using the two different buffers described. Our cytosolic results are therefore comparable to other groups using molybdate and our nuclear results are comparable to those of Walsh's group (1982), the only other workers attempting to measure non-extractable nuclear receptor.

As pointed out above, MTG has been found in earlier unpublished work in this laboratory to decrease the quantity of filamentous material in the nuclear preparations. Also, aggregation of nuclei is prevented by its use. The exclusion of MTG from the buffers used in nuclear isolation (because of its effect on the distribution of nuclear receptor (Walsh 1982) (see below)

therefore leads to both of these problems, but other steps in the purification process help overcome these drawbacks.

One step which contributed significantly to the purification process was the 10 minute extraction with 1 per cent Triton X-100. It has been shown by Kaufmann et al (1981), and others that nuclear membrane phospholipids and cytoplasmic contaminants are removed by detergents. Triton X-100 is a non-ionic surfactant which solubilizes cytoplasm, disrupts mitochondria, and removes the outer phospholipid layer of the nuclear membrane bilayer (Penman et al, 1966). This has the added effect of releasing nuclear membrane proteins which may be attached to cytoplasmic tags, thus rendering the nuclei free of such tags. Following this 10 minute incubation, centrifugation at 800 x g for 10 minutes pellets the nuclei, and the supernatant is discarded. Walsh (1982) has shown that nuclear receptors are not altered by this extraction treatment.

The pellet is then filtered through a wire mesh (the cheesecloth filtration step being abandoned), which removes any remaining bulky debris. The ultra centrifugation through 1.8M sucrose removes all mitochondria and remaining cytoplasmic debris from the nuclear preparation, leaving relatively purified nuclei for the extraction process.

Walsh's group (1982) as mentioned above, have found that the presence of DTT in the buffers alters the nuclear

receptor pattern, without affecting the total numbers. That is, the addition of DTT (or presumably another sulphhydryl reducing compound such as MTG or β -mercaptoethanol) to the buffers causes a marked increase in the extractable receptor levels (by much as 2-fold) when compared to extractable levels measured in the absence of DTT (Walsh, 1982). We may have noted an almost identical effect of MTG in our rat uterine studies, where we found that if the effect of MTG were presumably reversed by the oxidizing agent NaTT, the nuclear-extractable ER was decreased. The addition of NaTT to the extraction buffers was able to alter the ratio of extractable to non-extractable receptor from 70 per cent : 30 per cent (in the absence of NaTT), to 40 per cent : 60 per cent in its presence. These rat uterine tissues all were initially prepared in the presence of 100 mM MTG, the effect of which may have been reversed by the subsequent addition of NaTT, thereby altering receptor distribution so that nuclear ER were less easily extracted.

All published reports to date of extractable receptor levels in human prostate tissue have been measured in the presence of DTT, albeit low levels (approximately 1 mM), and for this reason, our first KCl extract results may not be strictly comparable. Our observed concentrations of AR (mean 178 fmols/g tissue) are approximately one third to half of those in other published reports; for example, the mean of 616 fmols/mg DNA in the latest report of

Trachtenberg et al (1982) and 512 fmols/g in Sirett's and Grant's recent report (1982) are higher than our values (see Table XI). In Trachtenberg's laboratory, average DNA in mgs per gram of tissue is 1 mg, thus his extractable receptor level would be approximately 600 fmols/g.

Interestingly enough, if our results for extractable receptor (1st extract) are expressed in fmols/mg of protein in the 1st KCl extract, then the mean value of 1st extract is 63 fmols/mg protein, a figure which is almost identical with earlier reports of Walsh and colleagues (Menon et al, 1978) (68 fmols/mg protein) and Shain and Boesel (1978) (60 fmols/mg protein). This is somewhat surprising since both of these groups were including DTT in their buffers at that time. Furthermore, both of these groups have since reported much higher levels, using the same methodology, but do not explain the increased levels in their more recent reports (Trachtenberg et al, 1981; Shain et al, 1980).

The problem of what parameters to use when expressing binding remains to be solved. The methods of protein estimation are generally standardized and reliable, such as the Bio.Rad method or Lowry method (1951). As mentioned above, this is calculated by measuring the protein concentration in the extract supernatant. Since our nuclear pellet is significantly purer than those of these other groups, our protein concentration is lower, so the value when expressed in fmols/mg protein would be expected

TABLE XI. Comparison of Nuclear Extractable AR Results
in Other Series

Authors	Mean Extractable AR Concentration (fmols/g tissue)
Trachtenberg et al 1982	600
Sirett and Grant 1982	512
Donnelly 1982	178

to be somewhat higher than values estimated from a crude pellet. On the other hand, in our nuclear isolation, thirty to fifty per cent of the nuclei are lost, which would lower the observed binding values. Likewise, Burton's method (1956) for DNA measurement is almost universally used. However, some groups take their samples for DNA assay from the initial homogenate, and this level will of necessity be higher than a sample measured just prior to KCl extraction, when some nuclei have inevitably been lost during purification.

Furthermore, measured prostatic DNA concentrations differ among laboratories, for example, Hicks and Walsh (1979) reported a mean of 1.9 mgs DNA/g of tissue; Trachtenberg (personal communication, 1982) reports a mean of 1 mg/g; Sirett and Grant (1982) a mean 1.2 mg DNA/g of tissue and our mean is 2.3 mg DNA/g of tissue. As pointed out above, the protein concentration of the nuclear extract will depend on the purity of the nuclei, which in turn will affect the values expressed in fmols/mg protein. In view of these difficulties, it is advisable for the present to express results using all three indices where possible until a satisfactory standard can be worked out.

The quantities of AR which were extracted by 0.6M KCl following DNase I digestion are considerably lower than the levels noted following the 1st KCl extraction (Table VIII). The fact that these receptors were only extracted

after the DNA was digested suggests that they are closely associated with the nuclear DNA. This may mean that there are two types of salt-insoluble or non-extractable receptors within the nucleus: one group is closely associated with DNA, and can be extracted once the DNA has been digested; the other group is closely associated with the nuclear matrix and is unaffected by DNase digestion. On the other hand, these results may simply be due to the fact that some salt-soluble receptors are trapped in the DNA within the nucleus and are only released following fragmentation of DNA.

There is accumulating evidence that the nuclear matrix-bound steroid receptor (or acceptor) sites are the biologically important receptor sites within the steroid target cells. Anderson, Peck and Clark (1973) have shown that the long-term retention of only a limited number of estrogen receptor complexes is a requirement for estrogen-induced uterine growth. They have subsequently shown that this number of sites is equal to the number of salt-insoluble sites in the nucleus (1975). Barrack and Coffey (1980) have suggested that these insoluble sites are associated with the nuclear matrix. Furthermore, these workers have shown that the levels of matrix-associated binding sites changes in response to manipulation of the hormonal status of the animals studied. For example, in the rat ventral prostate, the nuclear matrix-associated binding sites for DHT that are present in intact adults

essentially disappear within 24 hours of castration. Androgen replacement therapy restores, within 1 hour, the number of matrix binding sites for DHT to normal concentrations. Interestingly, these same authors point out that in the castrated animals, there were large amounts of AR in the cytosol when there were essentially none associated with the nuclear matrix, a finding that would call into question the significance of cytoplasmic assays, without simultaneous nuclear assays.

The report that dexamethasone-sensitive murine leukaemic myeloblasts contain salt-resistant nuclear binding sites for glucocorticoids, whereas the nuclei of certain clones of dexamethasone-resistant cells contain only salt-extractable hormone receptors (Honma et al, 1977), again suggests that these nuclear residual fractions may be involved in the responsiveness of normal and neoplastic cells to specific steroid hormones. Further evidence of the biological significance of matrix-bound receptors comes from the work of Sufrin et al (1982), who demonstrated in rat prostate that there is a 15 to 20 fold increase in the number of matrix-bound ER sites within 68 hours of induced prostatic growth.

The mean matrix-bound AR level in our study compares favorably to results reported by Walsh's group (1982) the only other group studying nuclear non-extractable receptors. They reported mean values of 250 fmols/mg DNA for non-extractable, and as noted above, their mean DNA

concentration per gram is 1.9. Therefore they noted AR at approximately 500 fmols/g of tissue in BPH. We noted a mean of 574 fmols/g of tissue in BPH. Since the nuclei in their preparation are not as pure as ours, it appears that the contamination with cytoplasmic debris does not affect non-extractable levels. However, their KCl-extractable levels are also approximately 500 fmols/g tissue (250 fmols/mg DNA). The total extractable AR in our series (sum of 1st and 2nd extract) is 252 fmols/g and strictly speaking, the values should be comparable because neither group used reducing agents during the isolation of nuclei. The difference between our procedure and that of Walsh's group (1982) is the purity of the nuclear pellet, ours being presumably much purer. This suggests that some of the extractable receptor Walsh's group (1982) are measuring may in fact be from the cytoplasmic debris.

The ratio of extractable to non-extractable nuclear AR in Walsh's study (1982) is 1:1 and in our study 1:2; Barrack and Coffey (1980) found a similar 1:2 ratio in their studies of the rat prostate nuclear receptors. These latter workers were assaying pure nuclei and using buffers identical to those of this present study, which again suggests that some of the extractable binding in Walsh's study (1982) may be from residual cytoplasmic debris.

The clinical significance, if any, of the matrix-bound receptors remains to be clarified by clinical

correlation in a series of patients, followed for a period of time. It would appear from Table VIII that while there is an overall 1:2 ratio of extractable to non-extractable nuclear receptors, there is considerable variation from tissue to tissue, which makes the measurement of matrix-bound receptors necessary for each individual tissue. The finding of higher mean levels of matrix-bound AR in normal tissue as compared to BPH tissue somewhat undermines Wilson's theory (1980) for the pathogenesis of BPH. One would expect to find higher levels of AR in the nuclei of BPH than normal tissue. The failure to establish this pattern leads one to question the importance of the finding that DHT levels in BPH tissue are two to four fold higher than in normal tissue. However, it is quite possible that when a larger number of normal specimens have been assayed, the mean for normal tissue will be lower than for BPH, since the levels of the three normals in this study fall well within the range seen in the BPH specimens.

The aims of this study were:

- 1) to measure cytosolic AR, ER, PgR and nuclear extractable receptor to see if a constant relationship between cytosolic, nuclear extractable and non-extractable receptors could be established;

- 2) to isolate nuclear matrices from human prostate tissue (normal, BPH and cancer);
- 3) to assay these matrices for AR.

I am happy to report that all three aims have been achieved, except that, due to shortage of adequate amounts of cancer tissue, nuclear assays in such tissue were not carried out. The numbers of samples assayed are not large, but only limited numbers of tissues were obtainable; also, a significant portion of the work was devoted to establishing the methodology. Now that the methods and preliminary studies have been successfully achieved, a larger series can be established and clinical studies carried out.

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